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The mobile ferrous iron pool in *Escherichia coli* is bound to a phosphorylated sugar derivative

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Based on *in vivo* Mössbauer spectroscopy it has previously been demonstrated that the intracellular iron pool of *Escherichia coli*, grown in iron deficient media supplemented with siderophores as the sole iron source, is dominated by a single Fe^{2+} and a single Fe^{3+} species. We have isolated the ferrous ion species and have purified it employing native column PAGE, chromatography and ultrafiltration. The purified compound displays an M_{app} of 2.2 kDa and an extremely low isoelectric point (p*I*) of 1.05. It is shown that this ferrous ion binding compound is neither a protein nor a nucleotide, rather it is composed mainly of phosphorylated sugar derivatives. This compound binds approximately 40% of the cytoplasmic iron. Therefore it is proposed that this oligomeric ferrous carbohydrate phosphate represents the long sought after mobile, low molecular mass iron pool.

Keywords: acyl carrier protein, ferrous iron, iron metabolism, mobile iron pool, phosphate esters, sugar derivatives

Introduction

Iron is an essential element for virtually all organisms and there is a growing body of knowledge about iron accumulation in microbial systems, plants and animals (Harrison & Lilley 1989, Grossmann *et al.* 1992, Theil & Hase 1993, Matzanke 1994a). Iron transport in many microorganisms is mediated by Fe^{3+} chelators (siderophores), which are excreted as desferri-compounds into the medium, and highly specific receptor and transport proteins located in the membranes and the periplasmic space (Winkelmann 1991, 1992, 1995, Matzanke *et al.* 1989b, Matzanke 1994b). The mammalian analog to siderophores is transferrin, which is transported into cells via endocytosis of the transferrin/ transferrin receptor system in an acidic, clathrin coated vesicle (Nunez *et al.* 1990). The main intracellular destinations of iron are also well known. On the one side there are the iron storage compounds, i.e. the ferritins, bacterioferritins and siderophores (depending on species) (Matzanke 1994a, Theil & Hase 1993), while on the other side are the whole breadth of iron containing enzymes and proteins into which the metal has to be transferred. However, what happens in between? How is the transported iron being processed? What is the nature of this intermediary iron? Up to now, all statements about this intermediary iron pool were speculative or at least very fragmentary.

Intracellular occurrence of a free Fe^{2+} hexaquo complex as an intermediary intracellular iron pool is extremely unlikely since both ferrous and ferric iron catalyze various reactions involving reduced forms of oxygen such as H_2O_2 and superoxide, i.e. Fenton chemistry or the iron catalyzed Haber Weiss reaction. The resulting hydroxyl radical reacts with almost every type of molecule found in living cells: sugars, amino acids, phospholipids, DNA bases and organic acids (Halliwell & Gutteridge 1984, Minotti & Aust 1987). Due to these reactions iron may participate in the oxidative damage of cells (Fridovich 1978, Halliwell & Gutteridge 1984) which has been hypothesized to be a causative factor in aging (Harman 1981), carcinogenesis (Harman 1981, Ames 1983) and to be a contributory factor in tumor promotion. In fact, it was demonstrated that oxygen free radicals generated by Fe^{2+} in aqueous solution are mutagenic (Loeb *et al.* 1988). Moreover, complexed forms of iron do not preclude *per se* reactions with activated oxygen species. Some chelates, e.g. the Fe–EDTA complex, are reactive in the iron catalyzed Haber–Weiss reaction, whereas others, e.g. ferrioxamine, are not (Halliwell & Gutteridge 1984, Dreyer & Dervan 1985, Basile *et al.* 1987, Inoue & Kawanishi 1987). Ferrous NADH and NADPH complexes also appear to act as Fenton reagents (Rowley & Halliwell 1982), as do AMP, ADP and ATP complexes (Rowley & Halliwell 1982, Floyd & Lewis 1983, Rush & Koppenol 1986).

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Although H_2O_2 and superoxide anions are mostly removed in aerobic organisms by catalase and superoxide dismutase, respectively, biological systems nevertheless produce detectable levels and so need to limit levels of available free iron (Griffith 1987). Therefore, virtually all organisms, growing either aerobically or anaerobically, must have evolved well designed iron containing enzymes, as well as low or high molecular mass iron transport and storage compounds which are sufficiently inert with respect to iron catalyzed Haber–Weiss–Fenton reactions. Moreover, stable intracellular ferrous iron carriers must be postulated which enable sheltered iron transfer (Matzanke *et al.* 1989a, 1991, 1992, Matzanke 1991).

In order to close the gap between iron transport, on the one side, and iron utilization and iron storage, on the other, the existence of a mobile, low molecular mass iron pool has been discussed controversially for many years (Tangeras *et al.* 1980, Crichton & Charloteaux-Wauters 1987, Bremond *et al.* 1988, Weaver & Pollack 1989, Crichton 1991, Fontecave & Pierre 1991). Some investigators have suggested that ATP- and GTP-bound iron are major constituents of the low molecular mass iron pool (Bartlett 1976, Mansour *et al.* 1985, Weaver & Pollack 1989). Taking into account the Fenton active nature of these compounds, this suspected role is very doubtful. Nilsen & Romslo (1985) assume that pyrophosphate serves as the intermediary transport ligand. However, there is a vanishingly small concentration of pyrophosphate in the cell and no evidence that sequestered sites with higher concentrations exist (Weaver & Pollock 1989). Other compounds which have been postulated as ligands for iron include amino acids, polypeptides, certain sugars and uncharacterized growth factors (Gessa *et al.* 1983, Tonkovic *et al.* 1983, Bakkeren *et al.* 1985, Wolowiec & Drabent 1985, Andersson & Porath 1986, Inoue *et al.* 1987). The weakness of these studies is that corresponding complexes either have not been isolated from biological sources or have not been identified as part of the mobile, low molecular mass iron pool. Moreover, it has been suspected that such a pool would represent only a tiny fraction of a percentage of the total cellular iron (Crichton 1991). Because of this the significance of such a pool has been questioned (Funk *et al.* 1986, Crichton & Charloteaux-Wauters 1987, Bremond *et al.* 1988, Crichton 1991).

Part of the controversy about the low molecular mass iron pool stems from the fact that ferrous ion compounds are difficult to analyze, because they are in general very sensitive to oxidation. In order to analyze the iron metabolism of microorganisms, and in particular the role of ferrous iron, we have developed an alternative approach, i.e. time resolved *in vivo* Mössbauer spectroscopy which allows us to monitor [^{57}Fe]siderophore transport and metabolization in iron depleted media (Matzanke 1987, 1991, 1994a, 1994b). Using this technique we could demonstrate unambiguously that in *P. agglomerans* siderophore iron is reduced intracellularly to the ferrous state and accounts for 95% of the cellular iron pool within 30 min of uptake (Matzanke *et al.* 1991). A Mössbauer spectroscopic analysis of *Escherichia coli* cells, grown to an $OD_{578}=0.8$ in media containing [^{57}Fe]ferricrocin as sole iron source, revealed two main components, a ferrous and a ferric iron species (Matzanke 1987, Matzanke *et al.* 1989a, 1992) each accounting for approximately 50% of the cellular iron pool. It could further be demonstrated that these same — or at least similar — components are found in many bacterial and fungal systems grown with siderophores as sole iron source (Matzanke *et al.* 1989a, 1990, 1991, 1992). Based on the Mössbauer spectroscopic data we have begun to isolate and characterize these ferrous and ferric iron species. While in an initial attempt we failed to uncover the nature of these two iron compounds (Matzanke *et al.* 1989a), we report here for the first time isolation and characterization of a soluble, low molecular mass ferrous iron complex which accounts for 40% of the cellular iron pool in *E. coli* under conditions of siderophore controlled growth.

Materials and methods

Chemicals

If not stated otherwise, all chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany). Isolation and purification of ferricrocin and preparation of desferriferricrocin have been described elsewhere (Wong *et al.* 1983). Purity of ferricrocin was checked by HPLC, performed on a Nucleosil reversed-phase C_{18} column [5 μm, 125 × 4.6 mm; gradient system: double distilled water/acetonitrile (6–40%, trifluoroacetic acid 0.1% (v/v), flow rate 1 ml min^{-1}; $r_t = 19$ min)]. Sephadex G-25, marker proteins (p*I* regions 2.5 6.5 and 3 10) and molecular mass peptides were purchased from Pharmacia Biotech Europe (Freiburg, Germany); Servalyt2-11, Servalyt2-4, Servalyt4-9T, Dowex 50 from Serva (Heidelberg, Germany); Coomassie brilliant blue R-250 and dithiothreitol (DTT) from Fluka AG (Buchs, Switzerland); and alkaline phosphatase (EC 3.1.3.1), TEMED and Trizma® base from Sigma (Deisenhofen, Germany).

Organism, growth conditions and separation of cell fractions

E. coli strain K12 W3110 was from the stock of the Institute. The strain was stored at −30°C in TY medium with 39% glycerol. *E. coli* cells were grown in iron depleted M9 medium supplemented with ferricrocin as specific iron source to late log growth phase ($OD_{578}=0.8$). The cells were then harvested and washed. Preparation of spheroblasts has been described elsewhere (Matzanke *et al.* 1989a). Spheroblasts were lysed, and the membrane and soluble fraction were separated by centrifugation.

In order to minimize oxidation of the ferrous iron compound during separation and purification all solutions were supplemented with 1 mM. In addition, the presence of oxygen was minimized by saturating all solutions with argon.

PAGE and isoelectric focusing (IEF)

Preparative native column PAGE was performed with a Bio-Rad preparative cell, model 491 [separating gel: 14% acrylamide, 0.4% bisacrylamide; electrode buffer: 25 mM

Tris, 192 mM glycine; sample buffer: 25 mM Tris, 0.1 mM DTT, 8.7% (v/v) glycerol, 0.004% tracking dye]. Electrophoresis of 10 ml of cytoplasmic fraction (148 mg protein, 24 μg Fe) was executed at a constant current of 40 mA. The elution chamber at the bottom of the electrophoresis column was separated from the electrode chamber by a cellulose dialysis membrane (MMCO: 3.5 kDa; Spectrum Medical Industries, Los Angeles, CA). Analytical Tricine–SDS–PAGE for low molecular mass compounds was applied according to Schägger & Jagow (1987) at a constant voltage of 100 V. Urea IEF was performed with a 2117 'Multiphor' chamber (LKB) using 'CleanGel' (Pharmacia) and ampholytes [Servalyt pH 2–4 (2%), Servalyt pH 3–10 (2%)] for 20 min at 500 V (8 mA, 8 W) and then 90 min at 2000 V (14 mA, 14 W). Silver staining was performed according to Blum *et al.* (1987). For preparative IEF we employed a 'Rotophor' preparative IEF cell (Bio-Rad, München, Germany), ampholyte solutions (Servalyt 2-4 and 4-9T at 2% (v/v)], 0.1 M H_3PO_4 anodic buffer and 0.1 M NaOH cathodic buffer solutions. IEF was performed at a constant power of 12 W for 5 h (final voltage of 790 V).

Ultrafiltration

Ultrafiltration under argon was executed employing an Amicon MMC ultrafiltration device and a Amicon UM2 membrane (MMCO: 2 kDa; Amicon, Beverley, CA).

Iron determination

Iron was determined either photometrically using the ferrozine method (Fish 1988) or via atomic absorption spectrometry (Perkin-Elmer atomic absorption spectrophotometer 400, 248 nm, 7 mA).

Phosphate determination

Phosphate was determined employing literature methods (Chen *et al.* 1956, Fiske & Subbarow 1925) which were slightly modified. Two grams of ascorbic acid, 0.5 g ammonium molybdate and 3.3 ml H_2SO_4 (95–97%) were mixed in a final volume of 100 ml distilled water directly before use. The sample (250 μl) was mixed with 1 ml of reagent and incubated for 2 h at 37°C. The A_{820} was then measured and compared with a reference (3 30 μg K_2HPO_4 ml^{-1}).

Sugar determination and characterization

Saccharides were determined photometrically using the anthrone reaction (Loewus 1952). The digoxigenin test for glycan conjugates (Boehringer, Mannheim, Germany) was also performed. Hydrolyzed and acetylated sugar derivatives were analyzed by GC-MS. For GC-MS, phosphorylated carbohydrate was hydrolyzed overnight in 1 N HCl at 100°C. The resulting solution was neutralized with saturated $Ba(OH)_2$ solution and the $BaCl_2$ precipitate discarded after centrifugation. The resulting sugars were reduced in aqueous solution with $NaBH_4$ in the dark overnight. Ion exchange resin (Dowex-50Wx8) was then added to eliminate excess reductant and the resin was removed by centrifugation. The supernatant was dried and redissolved in pyridine. After addition of acetic acid anhydride the solution was heated to 100°C for 1 h and after centrifugation was vacuum evaporated. The residue was redissolved in ethanol and dried again in vacuum. This procedure was repeated several times in order to remove residual pyridine. For GC and GC-MS the dried material was redissolved in $CHCl_3$.

GC-MS

The gas chromatograph was a Fractovap 2900 from Carlo Erba furnished with a HP-Ultra2 column. For separation a temperature gradient from 80 to 250°C (slope 4°C min^{-1}) was employed. Column 775 (PS255, glass capillary, $d = 0.5$ μm) was run on a Chrompack CP9001 at a temperature gradient from 150 to 240°C (slope 4°C min^{-1}). MS was executed on a Finnegan MAT 112 S. The Wiley-MS library enabled a comparison of the fragmentation pattern of the separated species with known compounds.

Protein sequencing

The N-terminal sequences of isolated proteins were analyzed via a gas phase protein sequencer (Applied Biosystems 477A).

Results

In order to separate, isolate and chemically characterize the ferrous iron species detected by *in vivo* Mössbauer spectroscopy we have performed a series of experiments including: (i) Cell fractionation. (ii) Separation of ferric and ferrous iron binding compounds using preparative column PAGE. This step also enabled enrichment and partial characterization of the ferrous ion binding compound (FIBCO). (iii) Further purification of FIBCO employing gel filtration and ultrafiltration. (iv) Characterization of FIBCO building blocks: (a) detection and determination of carbohydrate building blocks using spectrophotometric (anthrone reaction) and enzyme assays (DIG assay for glycan

Table 1. Distribution profile of iron in various cell fractions of *E. coli* K12 grown in iron depleted medium which was supplemented with fericrocin as the sole iron source

	Protein/mass wet cells (mg g^{-1})	Iron/mass wet cells (μg g^{-1})	Iron/mass protein (μg mg^{-1})
Cells	64 (100%)	18 (100%)	0.28
Spheroblasts	60 (93.8%)	17 (94.4%)	0.28
Cytoplasm	38 (59.4%)	13 (72.2%)	0.34
Membranes	19 (29.7%)	4 (22.2%)	0.21

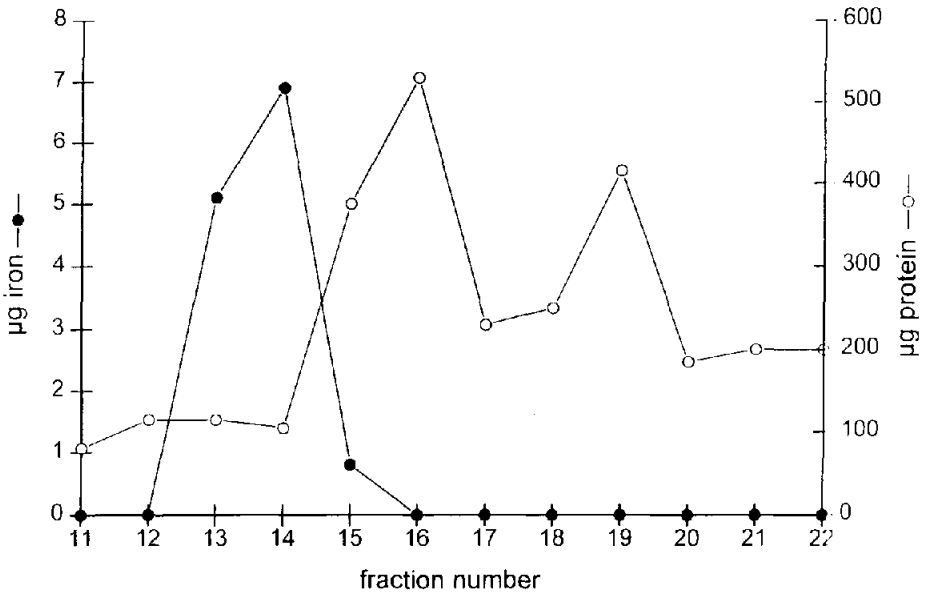

Figure 1. Elution profile of the cytoplasmic fraction of *E. coli* K12 W3110 (121 mg protein, 37 µg iron) on a preparative native column PAGE (see Materials and methods). Open circles correspond to protein and closed circles to iron.

conjugates) as well as GC and GC-MS, and (b) detection and determination of phosphate ester building blocks using IEF, colorimetric phosphate and enzymatic alkaline phosphatase assays.

Cell fractionation

Table 1 shows the distribution of iron in various cell fractions. Approximately 5.6% of the accumulated iron was found in the periplasmic space with 22.2% associated with the membrane fraction and 72.7% in the cytoplasmic fraction. For further separation and purification we chose the soluble, cytoplasmic fraction.

Separation of ferric and ferrous iron compounds and partial characterization of the ferrous ion containing compound

In an initial isolation step we could separate the ferrous iron component of the cytoplasmic fraction from the ferric by native preparative column PAGE. Figure 1 displays the elution profile from such a column. Fractions 13 15 contained 37.5% of the total iron applied to the column. Without addition of any external reductant fractions 13–15 gave the typical red complex with ferrozine indicative of the presence of *ferrous* iron. The corresponding Mössbauer spectrum exhibits within error bars the same Mössbauer parameters as the ferrous iron species in whole cells (Matzanke *et al.* 1989a, Böhnke *et al.* 1993). Based on a ferric/ferrous iron ratio of 1 (±0.1) in whole cells as determined by Mössbauer spectroscopy (Matzanke *et al.* 1989a, 1992), the yield of the ferrous iron containing compound after PAGE separation was approximately 75%. The remaining cytoplasmic iron, mainly corresponding to the ferric iron species, was retained on the column and could not be isolated by column PAGE. Biochemical and genetic characterization of this species is in progress and will be reported in due course.

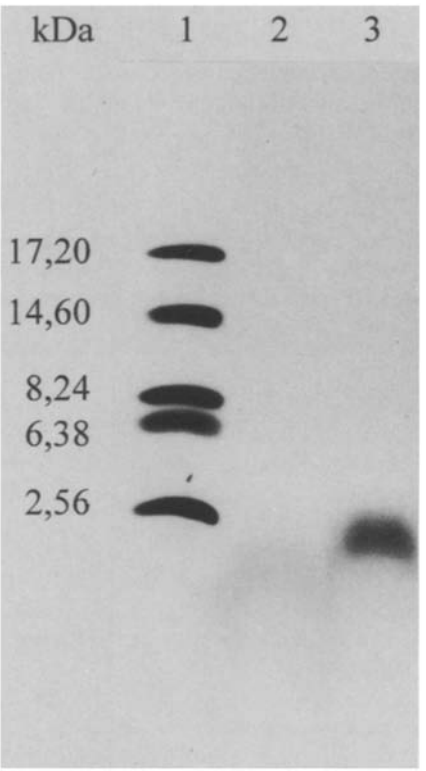

Figure 2. Silver stained Tricine–SDS–PAGE gel of fraction 14 (lane 2) and fraction 15 (lane 3) of the native preparative PAGE (see Materials and methods). Lane 1 represents marker peptides. On lane 2, a weak, single band of $M_{app} = 2.2$ kDa is visible and on lane 3 a single band at $M_{app} = 2.5$ kDa.

After silver staining, fractions 13 and 14 gave a weakly stained, broad single-band at 2.2 kDa on analytic Tricine–PAGE. Fraction 15 resulted in a well-stained but broad single band migrating at 2.5 kDa (Figure 2). Fraction

15 represents a protein, the N-terminal sequence of 27 amino acids of which was determined employing an automatic protein sequencer. Comparison with Swiss-Prot protein sequence database of EMBL (Heidelberg, Germany) revealed 100% homology to acyl carrier protein (ACP, 8.8 kDa) of *E. coli* (Vanaman *et al.* 1968). We determined the p*I* of the protein by IEF (p*I* = 4.0) (Figure 3). This result compares well with a reported p*I* value of 4.1 for ACP (Rock & Cronan 1982). Because ACP is not an iron protein we attribute the high amount of ferrous iron in the ACP fraction to an overlap with the ferrous iron containing compound of fractions 13 and 14.

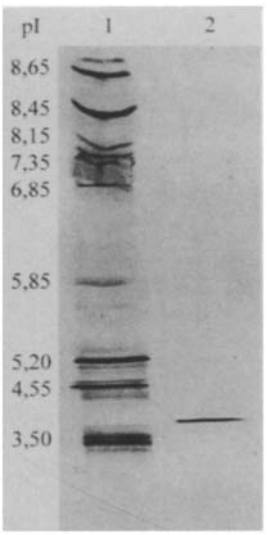

Figure 3. IEF gel (pH gradient 2–11) of fraction 15 (lane 2) and of marker proteins (lane 1). Only a single band at p*I* 4 is visible on lane 2.

Further purification of the ferrous ion binding compound

Fractions 13 and 14 contained material which gave a negative Coomassie but positive Lowry response. The material was also not detectable on a Tricine–PAGE via Coomassie staining and could not be sequenced. These results indicated the presence of a non-protein compound. In order to separate this ferrous ion binding compound from residual peptides, amino acids, nucleotides, etc., we employed Sephadex G-25 column chromatography (Figure 4) and ultrafiltration (fractions 15–21 of column chromatography). In ultrafiltration, the ferrous ion binding material was retained on a 2 kDa membrane. Silver stained Tricine–PAGE (data not shown) of the thus purified material yielded a single band at 2.2 kDa.

Characterization of the Fe^{2+} binding compound

Since the Fe^{2+} binding material was shown not to be a protein it was tested for the presence of carbohydrate. Fractions 13 and 14 of the preparative gel electrophoresis and fractions 15–21 of the gel filtration showed a positive digoxigenin-glycan assay. These fractions also exhibited a positive anthrone reaction. This reaction is sensitive to hexoses, pentoses and uronic acids. Absorption maxima were found at 513 and 561 nm, with a broad shoulder between 600 and 640 nm. For comparison we have obtained characteristic absorptions for anthrone derivatives of various monosaccharides, i.e. for aldohexose (glucose) and hexose (fructose) at 620–630 nm (bluish-green, $\varepsilon = 681$), pentose (ribose) at about 510 nm (brownish-green, $\varepsilon = 88.3$), and uronic acid (galacturonic acid) at about 550 nm (purple, $\varepsilon = 125.5$). It should be mentioned, however, that uronic acids exhibit an additional absorption maximum between 510 and 520 nm. Based on the extinction coefficients of the anthrone derivatives we determined a concentration of 7.5 mM ribose equivalents and/or 7 mM galacturonic acid equivalents and 0.8 mM glucose equivalents, i.e. the quantity of hexoses is an

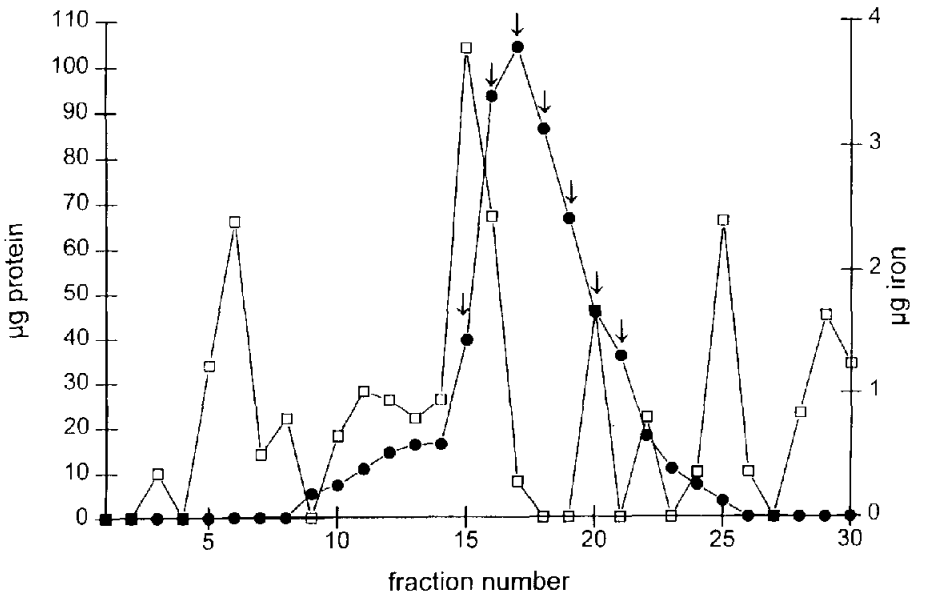

Figure 4. Elution profile of fractions 13 and 14 of the preparative PAGE on a Sephadex G-25 column. Open squares correspond to protein and closed circles to iron. Arrows indicate a positive digoxigenin-glycan assay and positive anthrone reaction.

order of magnitude smaller than that of the pentoses and/or uronic acids. Therefore, it is proposed that pentoses and/or uronic acids are the main building blocks of the Fe(II) binding compound. Since the ferrous ion concentration of purified material was measured to be 25 μM, the molar ratio of ribose equivalents/Fe(II) is 300, that of galacturonic acid equivalents/Fe(II) is 280 while that of glucose equivalents/Fe(II) is 32.

The carbohydrate building blocks of this compound were further analyzed by GC-MS following hydrolysis, reduction and acetylation. GC was performed on a HP ultra5 column yielding two major fractions with $r_t = 20.0$ and 26.8 min, and a minor one at 11.3 min. MS fragmentation patterns of these fractions were compared with a MS library (Wiley) and the peak at 26.8 min could be attributed to lyxofuranose, although the fit was not very good (fit score 570). Further GC, MS and NMR analyses are currently being performed in order to fully characterize the main building blocks of the novel cytoplasmic ferrous ion chelator.

The presence of nucleotides could be excluded since the UV-Vis spectra of these compounds after anthrone staining are clearly distinct from our ferrous ion chelator. Adenosine derived phosphate esters AMP, ADP and ATP exhibit absorption maxima at 495 nm with no absorption at higher wavelength. Moreover, GC of nucleotides prepared under the same conditions as applied for our novel ferrous iron chelator yielded different retention times (column 775: $r_{t\text{-PAGE}} = 6.3$, 10.6 min; $t_{t\text{-ATP}} = 13.2$, 14.0 and 15.8 min; $r_{t\ \text{NADH}} = 14.0$ min).

Finally, the novel ferrous ion binding saccharide derivative was further characterized by IEF which yielded a single band after silver staining at a remarkably low p*I* below 2 (Figure 5, lane 2). It is important to note that this band gave a positive ferrozine reaction indicating that the ferrous iron binds to this compound even at such a low pH, i.e. it is transported to the anode.

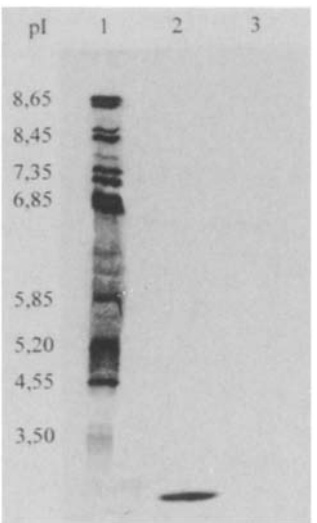

Figure 5. IEF gel (pH gradient 2–11) of fraction 13/14 (lane 2), of the same fractions after treatment with alkaline phosphatase (lane 3) and of marker proteins (lane 1). Only a single band at a extremely low p*I* below 2 is visible on lane 2. After treatment with alkaline phosphatase no band is visible on the IEF gel.

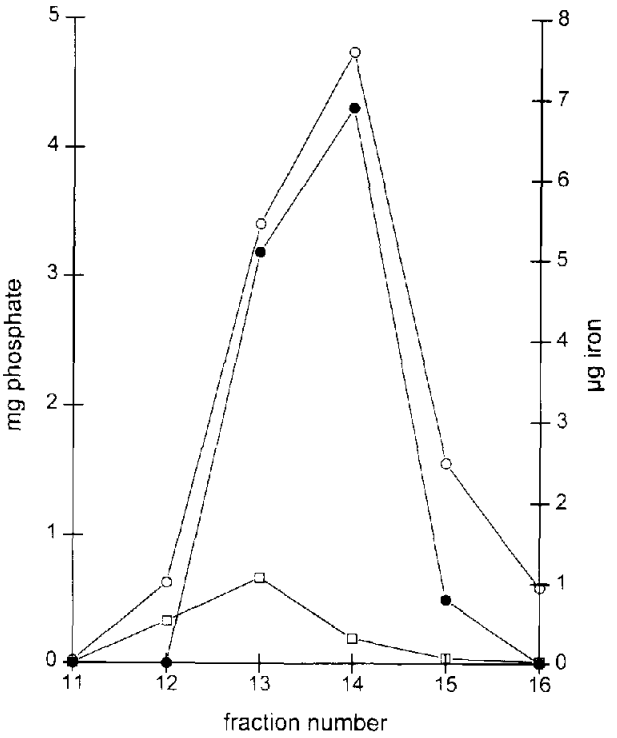

Figure 6. Fractions of preparative PAGE (see Figure 1). Closed circles correspond to iron and open squares indicate phosphate; open circles represent phosphate concentration in corresponding fractions after treatment with alkaline phosphatase.

For p*I* values below 2.5, there are no p*I* peptide markers available. Therefore, the p*I* of the iron binding carbohydrate was determined in a preparative IEF cell by measuring directly a pH of 1.05 in the segment containing iron and carbohydrate. Such an extremely low p*I* suggested the presence of negatively charged inorganic groups such as SO_4^{2-} or PO_4^{3-}. In fact, treatment with alkaline phosphatase results in disappearance of the band, as shown in Figure 5 (lane 3), providing evidence for the presence of phosphate esters. Colorimetric phosphate determination of preparative PAGE fractions yielded a phosphate profile different from the iron profile (Figure 6). However, the iron containing carbohydrate fractions 13 and 14 (and corresponding fractions 15–21 of gel filtration) exhibit high amounts of phosphate after treatment with phosphatase confirming the presence of phosphate esters in the Fe(II) binding saccharide derivative.

Discussion

For decades the existence of an mobile, intermediary, low molecular mass iron pool has been the subject of much

controversy since no such iron pool has ever been isolated. Only very recently, however, Laulhere *et al.* (1992) have shown that bulk iron in *Synechocystis* PCC 6803 is associated with low molecular mass compounds (< 10 kDa) of unknown composition. In this study, we provide the first evidence for a cytoplasmic, soluble, ferrous iron complex in *E. coli*. This compound binds approximately 40% of the cytoplasmic iron. Taking into account loss of iron containing material during purification, it corresponds well with the amount of ferrous iron in whole cells, which has been shown to account for approximately 50% of the total iron pool at late log growth phase (Matzanke *et al.* 1989a, 1992). The ligand exhibits an apparent molecular mass of 2.2 kDa. It is not a protein, but rather binds strongly to many proteins due to its negative charge(s). Two major constituents of this chelate have already been identified: (i) saccharides, the major one of which represents a pentose and/or an uronic acid, and (ii) phosphate esters. We suggest an oligomeric structure in which the monosaccharide building blocks are linked by phosphate esters and/or glycosidic bonds. In such a compound ferrous iron binding could be achieved either through sugar alcohol groups or phosphate. A discrimination between these two possibilities of ferrous ion binding, and a final determination of the building blocks and of the linkages requires an elucidation of the full structure. Corresponding work, including Mössbauer spectroscopic analysis, XANES, EXAFS, GC-MS, and ^{1}H-, ^{13}C- and ^{31}P-NMR, is in progress and will be reported in due course.

The function of this novel ferrous iron complex remains to be uncovered; however, it is likely that one of its functions is associated with the Fenton reaction. We expect that our novel sugar derivative will inhibit the Fenton reaction of ferrous iron. Moreover, this compound might play an important role in the biosynthesis of iron containing enzymes or in the reconstitution of the R2 subunit of ribonucleotide reductase (Fontecave *et al.* 1987, Karlsson *et al.* 1992). As earlier *in vivo* Mössbauer studies of a variety of microorganisms revealed iron components similar to those found in *E. coli*, we are convinced that this novel class of physiological ferrous iron binding agents is not restricted to *E. coli* or other Gram-negative bacteria but will also be detectable in Gram-positive bacteria, fungi and yeasts, and perhaps even in vertebrates.

In summary, we have detected a novel, ferrous iron binding, carbohydrate phosphate. We are convinced that this compound represents the long sought after mobile, low molecular mass iron pool. We expect that this novel ferrous ion complex is an anti-Fenton agent and an intracellular iron carrier used for various metabolic requirements.

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