Paraferritin: A Protein Complex with Ferrireductase Activity Is Associated with Iron Absorption in Rats†

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ABSTRACT: Recent studies reported that iron salts were absorbed in the duodenum utilizing a pathway involving membrane-associated integrin and a cytosolic protein named mobilferrin. In addition, a large molecular weight cytoplasmic complex was labeled with radioiron during mucosal uptake of iron in the duodenum. The molecular mass of this protein was 520 000 daltons, slightly larger than ferritin. On denaturing SDS−PAGE, the purified protein complex appeared to consist of at least four polypeptides, closely associated with each other. This complex was called paraferritin because its hydrodynamic volume resembled ferritin. In the present work, antibody studies demonstrate the presence of integrin, mobilferrin, and flavin monooxygenase in the water-soluble complex. Biochemical studies demonstrate the presence of a NADPH-dependent flavin monooxygenase ferrireductase activity that reduces Fe(III) to Fe(II). Antibodies against either integrin or mobilferrin inhibit monooxygenase activity. Inhibition of monooxygenase activity decreases radioiron uptake by tissue culture intestinal cells. Thus, we postulated that paraferritin plays a role in the mucosal uptake and transport of inorganic iron in small intestinal absorptive cells and is a mechanism for both the internalization of integrin from membranes to cellular cytosol and the delivery of iron to cellular constituents in an appropriate redox state.

Iron transport in the intestinal mucosa followed a novel pathway (Conrad & Umbreit, 1993a). Iron salts in the diet were initially chelated to intraluminal mucin (Conrad et al., 1991). The passage of iron across the cell membrane involved release from mucin and binding to a lumenal surface integrin in the membrane (Conrad et al., 1993a). Subsequently, the iron was transferred to a small cytosolic protein (56 000 daltons) called mobilferrin (Conrad et al., 1990a). A second cytosolic complex was labeled during iron transport and called paraferritin because of its molecular mass (Conrad et al., 1990b). Paraferritin is a large molecular mass protein complex (about 520 000 daltons) immunologically unrelated to ferritin. Although the existence of a large non-ferritin protein was suspected by ourselves and others (Pearson & Reich; 1969; Barton et al., 1978), this article constitutes the first description of the isolation and characterization of a 520 kDa iron binding protein complex.

Our studies show that paraferritin contains an integrin [approximate molecular mass on sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE)[1] for the α chain 150 000 daltons and β chain 95 000 daltons], mobilferrin (56 000 daltons), and a flavin monooxygenase (FMO, 60 000 daltons). Mobilferrin is highly homologous to calreticulin (Conrad et al., 1993b) which previously was shown to associate with rabbit lung flavin monooxygenase (FMO) and integrin (Conrad et al., 1993b; Guan et al., 1991; Rojiani et al., 1991). While the *in vivo* substrate for the family of FMO is not known, our results show that the paraferritin complex is capable of reducing Fe(III) to Fe(II) utilizing NADPH. Fe(II) is required in the cell as the substrate for ferrochelatase in the synthesis of heme.

We postulate these data have broad biological significance because ionic iron—unbound to protein—is believed to be involved in the etiology of several prevalent major catastrophic illnesses (Conrad, 1993b; Lauffer, 1992). Further, this is a demonstration of (1) integrin in aqueous cytosol, (2) a cellular mechanism for regulation of iron redox, and (3) a physiological nonpharmacological role for FMO.

EXPERIMENTAL PROCEDURES

Isolation of Paraferritin. Fifty Wistar, pathogen-free rats (175−200 g) were obtained (Charles River) and made iron-deficient by a 4-mL bleeding and placing them on an iron-deficient diet (17 mg/kg) (Teklad iron-deficient test diet with vitamin AOAC for rats; Madison, WI) and deionized−distilled water for 2 weeks before surgery. Iron-deficient rats were used to minimize apoferritin production within the mucosa and possibly stimulate production of substances which might be increased by iron deficiency. Rats weighed 240 ± 20 g and had a hematocrit of 35 ± 0.1% on the day of surgery. Under pentobarbital anesthesia (4 mg/100 g), a celiotomy was performed, and the duodenum was isolated with umbilical tape (pylorus to ligament of Treitz). Then 10 μCi of ^{59}Fe (Dupont, Billerca, MA) in 10 μM $FeCl_3$ (pH 2.2) was injected into the isolated duodenum. This was accomplished by puncturing the stomach with a blunted 18-g

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[1] Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FMO, flavin monooxygenase; NADPH, β-nicotinamide adenine dinucleotide; NTA, nitrilotriacetate; NADH, β-nicotinamide adenine dinucleotide; SOD, superoxide dismutase; methimazole, 2-mercapto-1-methylimidazole; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DS, dimethyl suberimidate; Ribi, monophosphoryl lipid [M:L] and trehalose dimycolate [TMD] emulsion; *Fenton reaction* (Fenton, 1894), iron-mediated free radical formation; PBS, phosphate-buffered saline (pH 7.4); BSA, bovine serum albumin.

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hypodermic needle, inserting it through the pylorus, tightening a ligature of umbilical tape, and injecting the test dose of radioiron and then withdrawing the needle and tying the tape. After a 10-min incubation, rats were perfused to death by rapid infusion of ice-cooled 0.15 M NaCl into the left ventricle following transection of the hepatic vein; viviperfusion markedly diminished contamination of mucosal preparations with hemoglobin and plasma transferrin. Isolated segments were excised, opened with an iris scissors, and washed thoroughly in two changes of cold 0.15 M NaCl. Mucosa was blotted with toilet paper to diminish mucin. Then mucosa was scraped from gut with glass slides on an iced glass plate at 4 °C. The scraped mucosa was placed in a solution of 0.15 M Hepes (pH 7.4) containing proteolytic inhibitors [benzamidine, 50 μg/mL; leupeptin, 1 μg/mL; phenylmethylsulfonyl fluoride, 75 μg/mL; and chymostatin, 5 μg/mL (Sigma Chemical Co., St. Louis, MO)]. Mucosal preparations were homogenized in a VirTis-23 homogenizer for 5 min (VirTis Co. Inc., Gardner, NY) followed by 12 strokes on a Potter−Elvehjem grinder with a Teflon pestle (Fisher Scientific Co., Atlanta, GA). Specimens were combined and centrifuged at 20000*g* for 30 min in a refrigerated centrifuge (Sorvall RC-5B, Dupont, Irving, TX). The supernatant was collected and treated as described below (Table 1):

Step 1. Protamine sulfate solution (2%, pH 7.4) was added at 4 °C with stirring to a final concentration of 0.2% protamine sulfate and incubated for 30 min at 4 °C. Then the mixture was centrifuged at 20000*g* for 30 min at 4 °C in a refrigerated centrifuge (Sorvall RC-5B, Dupont).

Step 2. Powdered ammonium sulfate was slowly added to the supernatant fluid with continued mixing on a magnetic stirrer to bring the final concentration to 60%. The mixture was maintained on a magnetic stirrer for 4 h at 4 °C. Then it was centrifuged at 20000*g* for 30 min at 4 °C. The supernatant contains mobilferrin, whereas the precipitate contains transferrin, ferritin, and paraferritin.

Step 3. The precipitate was washed in three changes of a 60% ammonium sulfate solution buffered with 10 mM Hepes at pH 7.4. Then it was resuspended in an aqueous solution of 10 mM Hepes (pH 7.4). The solution was heated at 70 °C in a water bath for 5 min. The mixture was cooled in an ice bath and centrifuged at 20000*g* for 30 min. The precipitate was discarded.

Step 4. Ultrogel ACA22 which was swelled in accordance with the instructions of the manufacturer (IBF Biotechnics, Columbia, MD) and placed in a 5 × 100 cm column was thoroughly washed with 0.15 M NaCl, 10 mM Hepes, and 10 mM EDTA (pH 7.4) in a chromatography refrigerator using gravity flow in a system connected to a LKB 2070 fraction collector (Bromma, Sweden) and UV monitor (Uvicord S2138). The supernatant from step 3 was applied to the Ultrogel ACA22 column, and the column was subsequently washed with buffer (0.15 M NaCl, 10 mM Hepes, and 10 mM EDTA, pH 7.4). Twelve milliliter aliquots were collected in the fraction collector using a drop counter. Radioactivity was quantified using a refrigerated automated γ detector (Packard Model 5230, Downers Grove, IL), and protein concentration was estimated in each test tube using a Gilford Response spectrophotometer (A^o 280 nm) (Ciba-Corning Diagnostics Corp., Medfield, MA) (Figure 1A). The test tubes containing radioiron-59 in the region of the column where ferritin (pre-transferrin peak) would be isolated were collected, and the contents were pooled.

Step 5. Preswollen cross-linked 5% agarose (lot 2004; Amicon Corp., Lexington, MA) was washed in 0.15 NaCl, 10 mM Hepes (pH 7.4) and placed in a 2.5 × 15 cm column. The selected material from step 4 was applied to the column and then the column was washed with 0.15 M NaCl, 10 mM Hepes (pH 7.4). Aliquots (12 mL) were collected using a fraction collector. The contents of tubes containing radioiron were pooled and placed in Spectra/Por 6.4 mL/cm dialysis tubing (MWCO 3500; Spectrum Medal Industries, Houston, TX). Dialysis was accomplished in three changes (8 h each) of a 20-fold excess of buffer (10 mM Hepes, 0.01 M NaCl, pH 7.4); this was required to rid the specimen of ammonium sulfate as tested using $BaCl_2$.

Step 6. DE52 (Whatman Biosystems, Ltd., Midstone, Great Britain) was washed in 0.01 M NaCl, 10 mM Hepes and packed in a 2.5 × 7.5 cm column. The dialyzed specimens from step 5 were applied to the column. Nine milliliter aliquots were collected using a drop counter. After washing with 0.01 M NaCl, 10 mM Hepes (pH 7.4) (200 mL), a gradient was begun using 0.5 M NaCl, 10 mM Hepes (pH 7.4) and a gradient former (Bio-Rad Model 365, Richmond, CA). Fractions containing radioiron following initiation of the gradient were collected and pooled (Figure 1B). The pooled radiolabeled material was placed in dialysis tubing for dialysis for 8 h with two changes of a 20-fold excess of 0.01 M NaCl, 10 mM Hepes (pH 7.4). In some preparations, two closely associated peaks of activity were identified on the DE52 column. These did not differ in specific activity of iron, FMO activity, ferric reductase activity, or bands on SDS−PAGE. They did differ in carbohydrate content based on Schiff−periodate staining.

Step 7. A DE52 column was prepared as in step 6 except that the column height was 1 cm. After the specimen was applied, the column was washed with 3 column volumes of 0.01 M NaCl, 10 mM Hepes (pH 7.4) buffer, and the protein was eluted using 0.5 M NaCl, 10 mM Hepes (pH 7.4). One milliliter aliquots were collected throughout, and the fractions containing radioiron following initiation of the saline wash were collected and pooled for analysis. For certain studies, the saline concentration was reduced immediately prior to use either by dialysis against 0.01 M NaCl, 10 mM Hepes (pH 7.4) buffer or by washing the specimen using this buffer in a Centricon-10 microconcentration tube (Amicon) and centrifuging the specimen at 7000*g* for 30 min at 4 °C.

In certain experiments, the isolate from the ion exchange column was chromatographed on an Ultrogel ACA-22 sizing column (100 × 1.0 cm), and the single radioactive peak (520 kDa) was isolated for studies.

Molecular Size. Molecular mass was estimated by gel filtration using Ultrogel ACA22 columns (1 × 100 cm) with comparisons to proteins of known molecular mass (Sigma). Estimates of the molecular mass of subunits were made by comparing the relative mobility of Coomassie (Bio-Rad)-stained bands to proteins of known molecular mass on fully denaturing SDS−PAGE gels.

Electrophoresis. Samples for electrophoresis were concentrated and desalinated with 10 mM Hepes (pH 7.4) by ultrafiltration using Centricon-10 tubes. Samples were concentrated sufficiently to permit addition of 100−200 μg of protein to sample wells. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) of reduced

and denatured samples was performed using 7.5% acrylamide in slab gels by the method of Laemmli (1970). Electrophoresis was performed in Bio-Rad dual electrophoresis cells with a Bio-Rad Model 3000/300 power source. Gels were stained with either Coomassie stain R-250 or Silver Stain Plus (Bio-Rad). Partially denaturing gels were prepared by the method of Laemmli, but the samples were not heated to 100 °C. Western blots were performed using fully denatured proteins on SDS−PAGE. Proteins were transferred onto nitrocellulose using a Bio-Rad Trans-Blot Cell according to the instructions of the manufacturer.

Antibodies. Polyclonal antibodies were raised in rabbits using purified isolates of mobilferrin (Conrad et al., 1990a); these reacted with both isolated and recombinant calreticulin (Conrad et al., 1993b, 1994). Polyclonal antibodies against integrins were raised in rabbits using isolations of rat integrin extracted from the 150 kDa protein bands on SDS−PAGE gels (Conrad et al., 1993a). This antibody reacts with the rat duodenal lumenal integrins which also reacted with α_3, α_5, and β_3 human monoclonal antibodies (Conrad et al., 1993a). Commercially obtained monoclonal antibodies (CD61) raised against rat β_3 integrin (PharMingen, San Diego, CA) and against human β_3 integrin (Becton Dickenson, San Jose, CA) were used to test reactivity with paraferritin. Antibodies against monooxygenase were obtained by immunizing rabbits against a synthetic peptide composed of a six amino acid (amino acids 299−304) sequence (KRRVKE) of monooxygenase (Guan et al., 1991). Western blots of whole cell extracts showed that the antibody of the peptide reacted exclusively with a protein of 60 kDa corresponding to FMO. Ribi (monophosphoryl lipid A [M:L] and trehalose dimycolate [TDM] emulsion; Ribi Immunochem Research, Hamilton MT) was used as an adjuvant for immunizing rabbits.

Tissue Culture. IEC-6 cells were obtained from the American Type Culture Collection (Rockville, MD). The cells were grown in monolayer cultures to confluence in 24 well Costar tissue culture plates (Cambridge, MA) using Dulbecco's modified Eagle's media with glutamine and 5% fetal calf serum (Whittaker M. A. Bioproducts, Walkerville, MD). Then the wells were washed twice in a Hepes−saline solution (10 mM Hepes, 0.15 M NaCl, pH 7.4), and Hepes−saline solution with 0.1% glucose and 2 mM $CaCl_2$ was added with various concentrations of inhibitor. Iron uptake was measured by adding 10^{-8} M iron-59 citrate (150 000 cpm/well) and incubating for various intervals. The iron citrate chelate effectively solubilized the ferric iron and permitted uptake by the alternate pathway of iron absorption as previously described (Conrad et al., 1994). Macromolecular complexes of ferric hydroxides (Spiro & Saltman, 1967) were not present as demonstrated by the failure of radioactivity to be sedimented by ultracentrifugation (134000*g* for 3 h). Subsequently, the medium was removed, the cells were rapidly washed in Hepes−saline solution, and the cells were solubilized in 0.5 mL of 0.5 N NaOH for measurement of radioactivity in an automated gamma detector.

The visible and ultraviolet absorbance of protein was measured in a Gilford Response spectrophotometer using a 1 cm light path. Protein elution from the columns was monitored by continuous measurement of the absorbance at 280 nm. Protein concentrations were determined by the BCA reagent (Pierce, Rockford, IL) using the absorbance at 562 nm.

Flavin Monooxygenase Activity. The activity of paraferritin associated with flavin monooxygenase was measured by the change in absorbance at 340 nm by the oxidation of NADPH. The reaction mixture contained 0.25 mM NADPH with 0.6 mM Fe−NTA (0.6 mM Fe and 2.4 mM NTA) in 900 μL of 20 mM Tris-HCl, pH 7.0. The buffer was equilibrated at 37 °C and the reaction initiated by addition of a 10 μL aliquot of purified paraferritin. The decrease in absorbance at 340 nm was determined in a Response II Spectrophotometer (Gilford Instruments, Oberlin, OH) and the rate determined with the best linear fit (standard deviation <0.010) for initial rates (less than 10% of substrate utilized). Heat-inactivated paraferritin, or paraferritin after prolonged storage at −40 °C, showed no activity. No attempt was made to chelate the product, ferrous iron, since ferrous iron salts are soluble at physiologic pH.

The reduction of ferric iron, Fe(III), to ferrous iron, Fe(II), by paraferritin and NADPH was also demonstrated by measuring the production of ferrous iron by the ferrozine method (Coves & Fontecave, 1993). In brief, an incubation mixture of 0.25 mM NADPH, 0.6 mM Fe−NTA, 2 mM ferrozine, 2.4 mM glucose 6-phosphate, and 2.61 units of glucose-6-phosphate dehydrogenase in a final volume of 900 microliters of 50 mM Tris-HCl (pH 7.5) was incubated with various amounts of purified paraferritin. The increase in absorbance at 562 nm due to the formation of the Fe(II)−ferrozine complex was determined at 35 °C for 5 min, and initial rates were determined.

Fluorescent spectra were monitored in a Perkin Elmer fluorescent spectrometer (Norwalk, CT). pH was measured at room temperature with a Chemcadet pH meter (Fisher Scientific Co).

RESULTS

Purification of Paraferritin. Rats were made iron-deficient by phlebotomy and diet to minimize ferritin production and maximize production of inducible iron binding proteins associated with iron absorption. Radioiron was injected into isolated duodenal loops *in vivo* with the animals anesthetized with pentobartital. Rats were killed by viviperfusion, and the duodenal mucosa was homogenized. Using heat, ammonium sulfate, and sizing and ion exchange chromatography, a large iron binding protein complex was isolated. On an Ultrogel ACA 22 column, the paraferritin complex eluted as a distinct shoulder (Figure 1A) of the larger radioactive peak of iron binding material (which contained predominantly transferrin). At this step of the purification, the paraferritin peak contained some residual ferritin as demonstrated by immunoblots. Subsequent steps removed the ferritin, and on DE52 chromatography, paraferritin eluted at the start of the salt gradient, separating it from the majority of remaining proteins (Figure 1B). The purification is summarized in Table 1. Both the large molecular weight iron binding protein and the FMO−NADPH oxidation activity were copurified by these procedures. Early steps removed other iron binding proteins (water-insoluble mucins and integrins and water-soluble mobilferrin, transferrin, ferritin, and non-protein-bound iron) such that the specific activity actually decreased but subsequent steps resulted in a progressive increase in the specific activity of the iron binding component. The iron bound to the paraferritin progressively dissociated from the protein complex over time

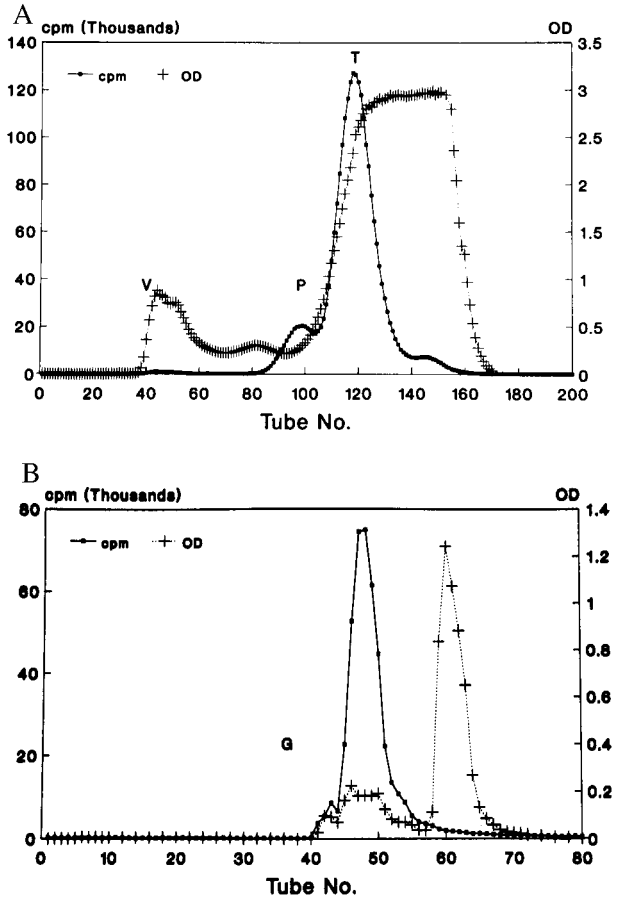

FIGURE 1: (A) Ultrogel ACA22 column showing exclusion volume (V), paraferritin (P), and transferrin (T). (B) DE52 anion exchange column isolates most of the radioiron in a small protein peak following initiation of the gradient (G).

in a pH-dependent fashion (Figure 2). Over about 72 h, approximately 58% of the original radioactivity was lost from purified paraferritin at pH 7.5. Since the purification procedure required about 4 days, and involved many procedures which would be anticipated to facilitate the dissociation of iron from paraferritin, the actual fold purification was probably considerably greater than indicated in Table 1. Similar technical factors affected the purification of FMO. Crude homogenates presumably had many NADPH oxidizing activities that were only partially corrected by including only Fe(III)-dependent NADPH oxidation. Removal of these competing activities resulted in a decrease in the apparent specific activity during the early purification steps.

Molecular Size of Paraferritin. The isolated protein was chromatographed on an ACA22 column in 10 mM Hepes, 0.15 M NaCl (pH 7.4). The hydrodynamic volume corresponded to a molecular mass of 520 000 daltons, slightly larger than ferritin. A single peak of protein was detected, coincident with the radioactive iron label. Autoradiographs and protein staining of both native and partially denaturing PAGE gels showed a single band which was radioactive. This band was detected in the gels about 1 cm from the point of application (Figure 3). Using SDS gels without heating the samples ("partially denaturing" PAGE gels) allowed the complex to remain intact and to retain the noncovalently bound radioactive iron. In contrast, heating the specimen led to loss of radioactivity and disruption of the complex with multiple bands being observed on fully denatured SDS−PAGE gels. SDS gel electrophoresis (fully denatured) showed five major bands: one at about 150 000 daltons, two bands at approximately 100 000 daltons, and two bands at approximately 60 000 daltons (Figure 4). The bands at about 60 000 daltons were very close and rarely visualized as separate bands (Guan et al., 1991). The staining with Coomassie blue of the band at 150 000 daltons was poor compared to silver staining. Since silver staining was not uniform for various proteins, the relative quantitative peptide composition could not be estimated from stained gels. The bands visualized on SDS gels were Schiff positive, indicating that varying degrees of glycosylation were present, and this contributed to the somewhat diffuse character of the bands. Treatment with dimethyl suberimidate, a protein cross-linking reagent, resulted in a disappearance of these bands, and the intensification of a protein staining material that barely entered the gel (Figure 4). This showed that the various peptides were in close association to each other as part of a single multiple peptide complex (Packman & Perham, 1982; Davies & Start, 1970). Similarly, ferritin showed the expected bands at 18 500 and/or 22 000 daltons which become cross-linked upon treatment with dimethyl subermidate to a protein complex which barely entered the gel. Cross-linked proteins such as paraferritin and ferritin would not be expected to enter the gel significantly, because they would not be denatured in SDS to small molecular sized components.

Although minute membrane fragments should have been excluded from the specimen on the Ultrogel ACA22 column at the void volume, paraferritin was centrifuged in a Beckman Airfuge (Palo Alto, CA) at 134000*g* for 60 min to provide greater assurance that the iron binding proteins were soluble in the aqueous solution. Starting with 104 803 cpm, the supernatant after centrifugation contained 97 707 cpm and the pellet 2007 cpm. Only 2% of the counts were found in the pellet, so that it is unlikely that the radiolabeled paraferritin complex was associated with either vesicle or membrane fragments. Since paraferritin and free iron bound to glass and plastic with some avidity and the washed tube was radioactive, the 2% in the pellet was an overestimate. Using the BCA method (Pierce) for measuring protein, there was 328 μg/mL before centrifugation versus 320 μg/mL in the supernatant postcentrifugation.

Integrin, Mobilferrin, and Monooxygenase Were Paraferritin Subunits. In order to demonstrate the component proteins in paraferritin, the complex was reacted with antibody to the individual components. Immunoblots were prepared containing native paraferritin (Figure 5A). The native paraferritin was reactive to antibodies produced against purified rat integrins (Conrad et al., 1993a), mobilferrin (Conrad et al., 1990a), and the FMO peptide (Experimental Procedures). Since certain integrins bind fibronectin, the capability of purified paraferritin to bind fibronectin was studied. Binding of fibronectin to paraferritin was documented on immunoblots (Figure 5B). There was no reactivity to ferritin and transferrin antibodies. Commercially obtained monoclonal antibodies raised against both rat and human β_3 integrin subunits also showed reactivity with paraferritin on immunoblots (not shown). Western blots transblotted from fully denatured SDS−PAGE gels showed reactivity with a polyclonal antibody raised against an α subunit of rat intestinal cell integrin (150 kDa) which has

Table 1: Purification of Paraferritin[a]

step	iron binding activity			FMO activity		
	cpm/mg	yield (%)	*x*-fold purification	*A* (min mg^{-1})	yield (%)	*x*-fold purification
crude homogenate	12224	100		0.045	100	
crude supernatant	8357	29	0.68	0.044	41.7	0.97
protamine sulfate	7329	27	0.51	0.40	40.7	0.90
ammonium sulfate	11088	23	1.28	0.038	10.0	0.84
heat	15745	16	1.28	0.038	10.0	0.84
ACA22	97692	1.6	7.9	1.79	12	39.0
agarose	120296	1.6	9.8	1.85	7	41.0
DE-52	238670	0.8	19.5	12.00	10	266.7

[a] Rat mucosa was labeled *in vivo* by the infusion of radiolabeled iron into the duodenum as described under Experimental Procedures. After a 10-min incubation, the duodenum was isolated and homogenized, and paraferritin was purified by the indicated steps (first column) monitoring the *in vivo* label (second column). The label was expressed as cpm/mg of protein reflecting the specific activity. The iron was reversibly bound to paraferritin and dissociates during the purification (cf. Figure 2), so that the yield (third column) and fold purification (fourth column) were underestimates. In addition, prior to step 6, there were several other iron binding proteins present (Conrad & Umbreit, 1993) so that the specific activity and yield in the early steps were not solely due to paraferritin. In parallel samples, the FMO-related activity was measured as the ferric iron dependent NADPH oxidation, by the change in absorbance at 340 nm (fifth column) expressed as a specific activity. The FMO activity was purified in parallel to the *in vivo* radioactive iron label, and the yield (sixth column) and fold purification (seventh column) were determined.

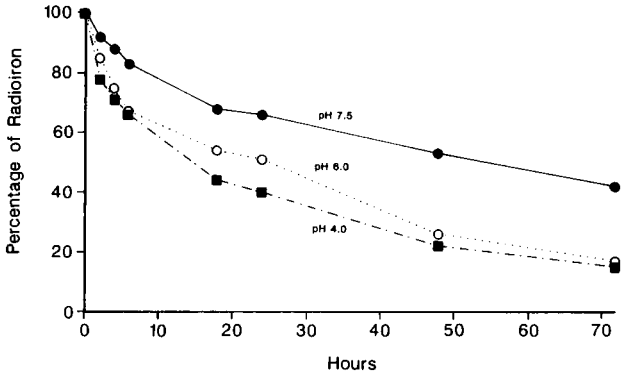

FIGURE 2: Dissociation of iron from paraferritin. Purified paraferritin (274 064 cpm) was placed in a dialysis bag (Spectra Por 6, Spectrum, Los Angeles, CA) and dialyzed against 10 mM Hepes, pH 7.5 (closed circles), 10 mM Hepes, pH 6.0 (open circles), and 10 mM sodium acetate, pH 4.0 (closed squares). Each buffer also contained 0.1 mM NTA. After the indicated time, the dialysis tubing was removed, and the counts remaining in the retentate were determined and calculated as a percentage of the radioactivity in dialysis bags prior to incubation.

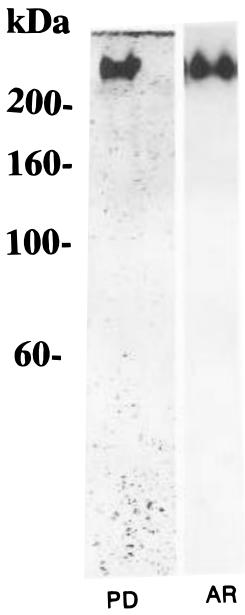

FIGURE 3: Homogeneity of paraferritin by gel electrophoresis. Paraferritin was applied to a partially denaturing SDS−PAGE gel and electrophoresed (left lane). Silver-Stain Plus revealed a single band about 1 cm from the point of entrance into the gel. The location of the bands on the gel suggested the radioiron was associated with a large complex; quantitative measurements of molecular size could not be obtained on these gels because they were only partially denatured. Autoradiography of a duplicate lane from the gel showed the band contained radioiron (right panel) coincident with the location of the protein band.

been associated with iron absorption (Conrad et al., 1993a) (Figure 6).

Kinetic Parameters Associated with Paraferritin. Employing standard methodologies, the pH optimum for NADPH oxidation and K_m values for Fe(III) and NADPH were determined. Using methimazole (2-mercapto-1-methylimidazole) as a substrate and measuring the oxidation of NADPH, activity was demonstrated with a pH optimum of ~pH 8.5, similar to that described for rabbit lung FMO (Guan et al., 1991). In addition to artificial substrates such as methimazole (a commonly used substrate for FMO), paraferritin utilized Fe(III)−NTA (iron−nitrilotriacetate chelate) as a substrate. This reaction had a broad pH optimum of approximately pH 7.0 (Figure 7). Lineweaver−Burk plots of the reaction demonstrated a K_m for NADPH of 74 μM. The V_{max} for NADPH was 42 nmol min^{-1} mg^{-1}. Since the FMO component comprised only a portion of the complex (60/520), this corresponded to a rate of 364 nmol min^{-1} mg^{-1} for the FMO subunit. This rate was comparable to other substrates utilized by FMO (Coves & Fontecave, 1993; Williams et al., 1985). The K_m for Fe(III)−NTA was measured as 2.1 mM. NTA−Fe(III) is in equilibrium with a limited concentration of free iron which would be the effective substrate for paraferritin. The dissociation contant for paraferritin with respect to free iron was calculated[2] to be approximately 2.6×10^{-15} M. Such tight binding would be required to avoid concentrations of iron that might catalyze the *Fenton reaction* (Fenton 1894; Chance et al., 1979) resulting in free radical damage to the cell. Alternatively, *in vivo* the iron might bind mobilferrin (effective dissociation contant about 3×10^{-18} M) and subsequently bind to the peptides in paraferritin.[2]

Flavin Content of Paraferritin. The fluorescent spectra of isolated paraferritin showed excitation at 380 and 450 nm and an emission at 509 nm. The absorbance spectrum was similar to that reported for FAD containing rabbit lung FMO,

[2] Personal communication graciously provided by Philip Aisen, Albert Einstein College of Medicine.

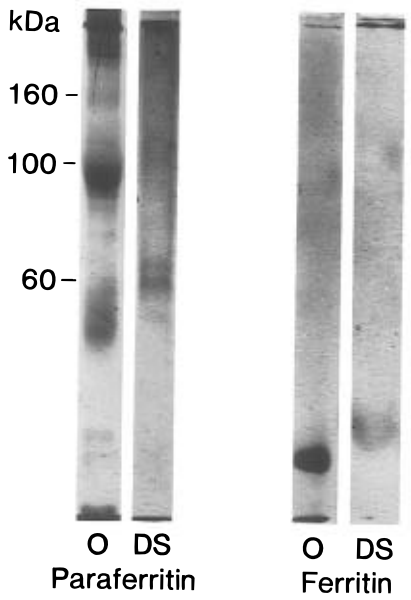

FIGURE 4: Cross-linking of subunits of paraferritin and ferritin with dimethyl suberimidate (DS)(Davies & Stark, 1970). The left panel shows the results with paraferritin. The first lane shows SDS−PAGE of untreated paraferritin with bands at 150 000 daltons and 90 000 daltons, a doublet at 60 000 daltons, and two small bands. Some partially denatured protein remained at the top of the gel. The same quantity of paraferritin was reacted with DS in the second lane prior to electrophoresis. In this reaction, a protein sample of 100 μL of 3 mg/mL paraferritin was incubated with 10 μL of DS (60 mg/mL in 0.2 M ethanolamine, pH 8.0) for 2 h at room temperature. Then SDS sample buffer was added and the sample heated at 100 °C for 10 min and electrophoresed. The right panel shows the same experiment utilizing the same amount of ferritin. The first lane shows SDS−PAGE of untreated ferritin, demonstrating a band at 18 500 daltons, and the second lane shows ferritin treated with DS prior to electrophoresis.

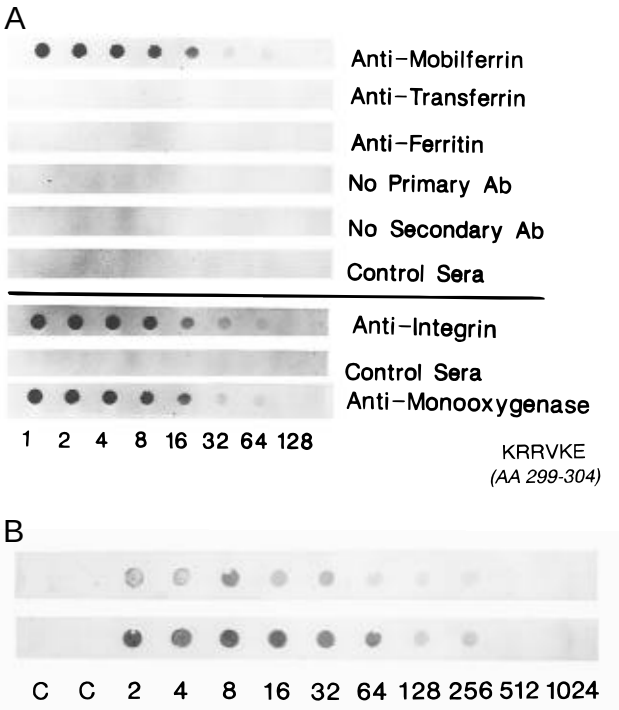

FIGURE 5: Immunoblots demonstrate the component proteins in paraferritin. Components of paraferritin (A). Paraferritin was reacted with antibodies raised against rat α integrin (Conrad et al., 1993a), mobilferrin (Conrad et al., 1990a), and a FMO-specific peptide (Experimental Procedures). Native paraferritin was blotted onto Immobilon filters (Millipore, Bedford, MA). One hundred microliters of a solution of purified paraferritin (20 μg/mL) was applied to the first well of a Bio-Rad Dot Blot apparatus with serial dilutions in successive wells as indicated. The numbers are the reciprocal of the dilution. After washing and blocking with dehydrated nonfat milk, the primary antibody or control serum was utilized on individual strips. The secondary antibody was goat anti-rabbit (for rabbit polyclonal antibodies) or goat anti-mouse (for monoclonal antibodies). The secondary antibody was gold-labeled and enhanced by silver stain according to the manufacturer's directions (Bio-Rad). The antisera used were against rat mobilferrin (rabbit, polyclonal, 1:250), rat transferrin (monoclonal, 1:500), ferritin (monoclonal, 1:500), β_3 integrin (1:500), and monooxygenase (1:500). In the native paraferritin, mobilferrin, integrin, and FMO were detected, but ferritin and transferrin were not. No reactivity was observed either with normal rabbit serum (1:250) or in the absence of either primary or secondary antibody. Fibronectin binds paraferritin (B). Purified paraferritin which was ultracentrifuged was diluted serially on a Dot Blot apparatus onto Immobilon. The Immobilon was blocked with 5% nonfat milk in PBS for 2 h. This was washed in three changes of PBS and incubated for 2 h with 10 μg/mL human fibronectin (Sigma) in 1% BSA in PBS. The Immobilon was washed 3 times with PBS and incubated for 2 h with a monoclonal antifibronectin antibody (Sigma). This was washed with three changes of PBS and incubated with either goat anti-mouse conjugated with alkaline phosphatase (upper row) or goat anti-mouse labeled with colloidal gold (lower row) (Bio-Rad). The contents of the lower row were subsequently enhanced with silver (Bio-Rad). Each secondary antibody was diluted in 5% BSA in PBS and incubated for 2 h. Blots were washed 3 times, and color was developed with Bio-Rad reagents. Controls (C) lacked either fibronectin or primary antibody, respectively.

and the emission was characteristic of a flavin moiety (Guan et al., 1991). In order to identify the flavin moiety more precisely, the flavin was released by acid from purified paraferritin and analyzed by reverse-phase high-pressure liquid chromatography (Light et al., 1980). The absorbance at 450 nm was utilized to detect the flavins. FAD was detected contaminated with less than 10% FMN (Figure 8). For each mole of paraferritin, approximately 1.2 mol of FAD was released. FMO has previously been determined to have 0.91 mol of FAD per mole of enzyme as the only flavin, and this was quantitatively released by acidification (Ziegler & Mitchell, 1972).

Monooxygenase Activity of Paraferritin. When presented with a substrate such as iron which cannot be hydroxylated, paraferritin, like other monooxygenases, produced hydrogen peroxide by donating an electron to oxygen. Paraferritin extracted electrons from NADPH to form superoxide ($O_2^{\bullet -}$). This was demonstrated by the superoxide dismutase (SOD) inhibited reduction of cytochrome *c* (McCord & Fridovich, 1969). Unlike many FAD-containing enzymes, paraferritin was unable to reduce cytochrome *c* directly. The superoxide then dismutated to hydrogen peroxide. The rate of hydrogen peroxide production by paraferritin (14.7 nmol min^{-1} mg^{-1}) was half the rate of NADPH oxidation under identical conditions, indicating that one electron from NADPH was donated to oxygen. The other electron was used to reduce ferric iron. Utlizing Fe(III)−NTA as substrate, paraferritin oxidized NADPH to reduce Fe(III) to Fe(II) detected as the Fe(II)−ferrozine chromogen (Figure 9). This confirmed that during the ferric iron dependent oxidation of NADPH the product was indeed ferrous iron. Ferrozine complexed with ferrous but not ferric iron to form a specific chromogen. Ferrous iron was produced in a time- and concentration-dependent manner. The activity was destroyed by heating paraferritin at 100 °C for 5 min. For every two electrons donated from NADPH, approximately one electron was used to reduce Fe(III).

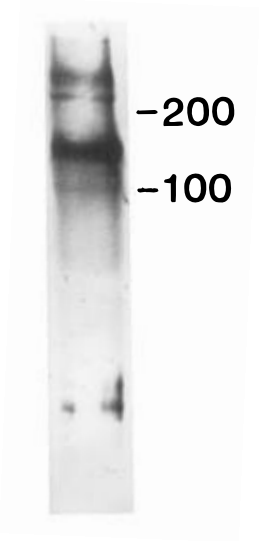

FIGURE 6: Western blot for detection of the α subunit of a rat integrin in purified ultracentrifuged paraferritin. Paraferritin was precipitated by 80% ethanol and resuspended in SDS sample buffer. Proteins were electrophoresed on SDS−PAGE 7.5% acrylamide gels and transblotted onto nitrocellulose. The nitrocellulose was blocked with 5% nonfat milk in PBS and then washed in three changes of PBS. It was incubated for 2 h with a 1:250 dilution of rabbit anti-rat intestinal α integrin in 1% BSA and PBS. The Immobilon was washed in PBS, incubated with goat anti-rabbit conjugated alkaline phosphatase, and developed according to manufacturer's instructions (Bio-Rad). A dense staining band was observed in the 150 kDa region with a weakly staining band at 90 kDa. Some staining was observed in the gel near the point of application, which was believed to represent undenatured paraferritin.

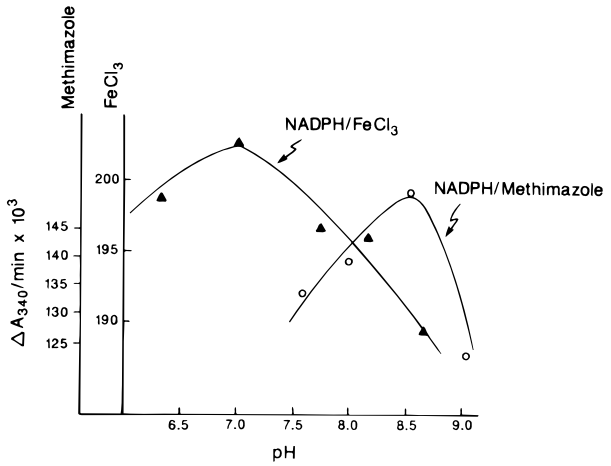

FIGURE 7: pH optimum of oxygenase. The assay utilized 0.1 mM methimazole, 0.025 mM NADPH in 1 mL of 2 mM Tris-HCl at the appropriate pH. Enzyme was added (40 μg) and the change in absorbance at 340 nm determined. For experiments with Fe(III) reduction, 160 μg of enzyme was utilized with 1 mM $FeCl_3$ (chelated as the NTA salt). Incubation was at 37 °C. Activity with NADH showed the same pH profile as with NADPH but with 30% lower activity.

Inhibition of FMO Activity of Paraferritin by Antibody to Integrin and Mobilferrin. Paraferritin was incubated with various concentrations of antibody to integrin (Figure 10) and mobilferrin (Figure 10). In each experiment, a significant decrease in the ability to oxidize NADPH was observed with increasing concentrations of the specific antibody. This implied that the integrin and mobilferrin directly interacted with the FMO subunit.

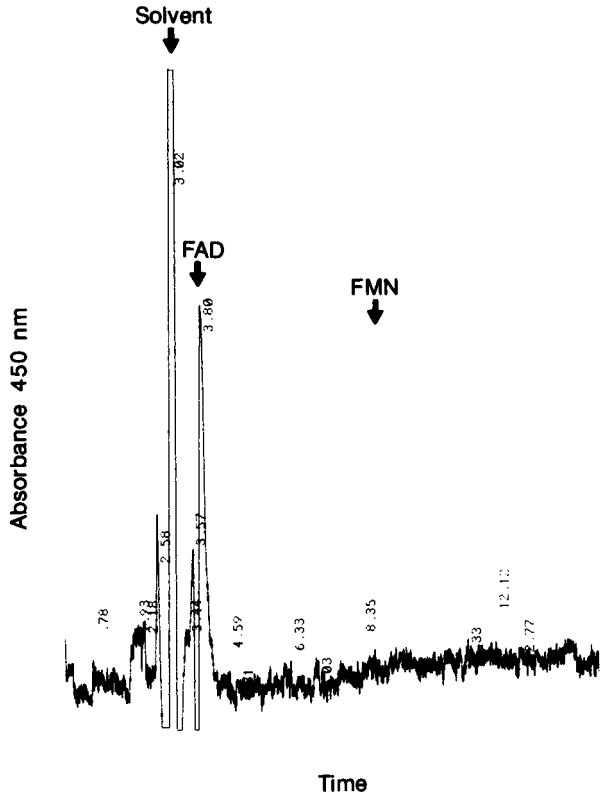

FIGURE 8: HPLC of flavin moiety from paraferritin. Purified paraferritin (510 μg) was concentrated by precipitation with 80% ethanol at 4 °C for 2 h and collected by centrifugation in a microcentrifuge (Fisher Model 235-C, Pittsburgh, PA) for 10 min. The pellet was air-dried for 5 min and resuspended in 90 μL of distilled water, and 7 μL of 70% perchloric acid was added at 4 °C. This was immediately centrifuged in the microcentrifuge for 3 min; the supernatant fluid was removed and neutralized with 21 μL of a suspension of potassium bicarbonate (12 g in 10 mL of H_2O). This was centrifuged twice in the microfuge, each time for 10 min. The resulting supernant (90 μL) was analyzed by HPLC reversed-phase chromatography (Light et al., 1980). A Bonapak C-18 column (0.39 × 30 cm; Millipore, Bedford, MA) was used with a mobile phase of 30% methanol and 70% 5 mM ammonium acetate, pH 6.0, in H_2O. The eluent was monitored by the absorbance at 450 nm (Waters Model 450 variable-wavelength detector). The retention times were compared to authentic samples of FAD (3.8 min) and FMN (8.04 min) (Sigma Chemical Co.) and were integrated by a Spectra-Physics Chromjet Integrator (Model SP4400, San Jose, CA). In the sample from paraferritin, only the solvent and salt peak (3.02 min) and a peak corresponding to FAD (3.80 min) were detected.

Effect of Phenelzine on FMO and Iron Uptake. Various compounds were investigated to determine if they were capable of inhibiting the reduction of ferric iron by paraferritin. Increasing concentrations of phenelzine inhibited the ability of paraferritin to reduce Fe(III) as measured by oxidation of NADPH (Figure 11). The effect of phenelzine upon iron uptake by cells was tested in a tissue culture of IEC-6 rodent intestinal cells. Phenelzine inhibited iron uptake probably due to its effects upon flavin monooxygenase (Figure 11), since there was no change in cell viability as determined by trypan blue exclusion. The enzyme was inhibited at about 25 mM phenelzine, whereas IEC-6 cells showed a decrease in iron uptake at 5 mM phenelzine. Phenelzine appeared to inhibit Fe(III) reduction by functioning as a competitive substrate of low reactivity. Thus, the degree of inhibition by phenelzine would depend on the concentration of the substrate (iron) which will be different *in vivo* compared to *in vitro*. The inhibitory concentration

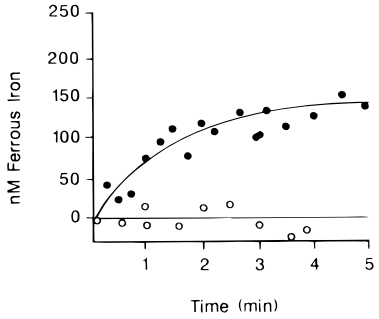

FIGURE 9: Reduction of Fe(III) to Fe(II) by paraferritin. The activity of paraferritin (0.3 μg) was measured as described under Experimental Procedures utilizing a NADPH regenerating system, using Fe(III)−NTA as substrate, and detecting the formation of Fe(II)−ferrozine (Coves & Fontecave, 1993). Ferrous iron was produced in a time-dependent reaction (closed circles), and the Fe(II) produced was expressed as nanomolar based on the absorbance of Fe(II)−ferrozine at 562 nm (E = 28 mM^{-1} cm^{-1}) not corrected for the effect of NTA. Paraferritin which was inactivated by heating at 100 °C for 10 min prior to assay did not demonstrate activity (open circles). Addition of 15 μM riboflavin did not increase ferrous iron production. The NTA in the reaction mixture used to solubilize the Fe(III) was the same chelator used when oxidation of NADPH was determined. The NTA, however, interfered with the color development with ferrozine. Since less than 10% of the substrate was consumed under the conditions used, the concentration of free NTA was minimally altered during the reaction. Since the concentration of NTA was therefore known, the inhibitor effect on ferrozine chromogen formation could be corrected. After this correction, it was shown that 0.796 nmol of Fe(II) was produced for the same amount of enzyme and time that oxidized 0.625 nmol of NADPH. The oxidation rate of NADPH was determined in independent reactions in the absence of ferrozine. The ratio of Fe(II) produced per moles of NADPH oxidized was 1.3. This was close to the expected ratio of 1.0 that would occur if one of the electrons donated from NADPH was used to reduce Fe(III) to Fe(II).

of phenelizine would not be expected to be the same for the purified enzyme compared to *in vivo*. These data suggested a direct role of paraferritin and the reduction of iron in the transport of iron via the alternative pathway (Inman et al., 1994).

DISCUSSION

Iron was transported from the intestinal lumen by the integrin−mobilferrin pathway (Conrad & Umbreit, 1993a). Iron in the diet was initially chelated to mucin at an acid pH for delivery to the small intestine in a soluble and absorbable form (Conrad et al., 1991). The iron was transported through the membrane in association with an integrin (Conrad et al., 1993b). Subsequently, a small molecular mass (56 000 daltons) protein, mobilferrin, and a large (520 000 daltons) complex protein, paraferritin, were radiolabeled. In this study, it was shown that paraferritin (520 kDa) contained mobilferrin (56 kDa), flavin monooxygenase (60 kDa), and water-soluble integrin (~250 kDa). There were either more than a single molecule of these components in paraferritin or additional components because the sum of the molecular masses of the identified proteins was significantly less than 520 kDa. SDS−PAGE gels of fully denatured paraferritin suggested there may be other components which have not yet been identified.

Flavin monooxygenase (FMO) was demonstrated by reactivity of paraferritin with an antibody raised against a hydrophilic amino acid sequence of monooxygenase and its capability to oxidize NADPH with classic substrates such

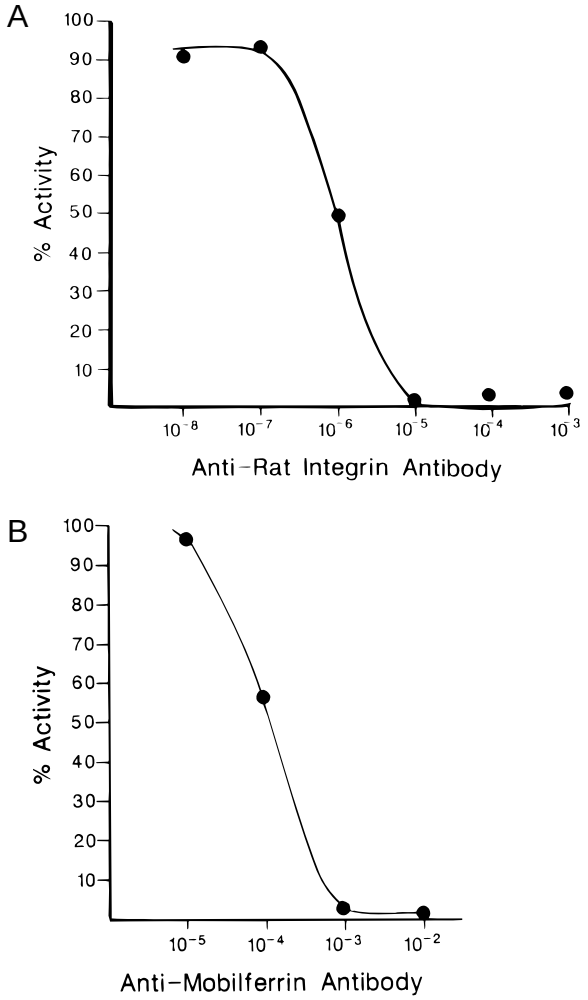

FIGURE 10: Inhibition of paraferritin monooxygenase activity by antibody. Antibody to rat integrin (A). Antibody to rat integrin was added at the final dilution indicated in 1 mL of a mixture of 20 mM Tris-HCl, pH 7.5, 160 μM NADPH, 0.6 mM $FeCl_3$, and 2.4 mM NTA. To this was added 42.5 μg of purified paraferritin and the decrease of absorbance at 340 nm was determined. A control lacking paraferritin was subtracted from the resulting value, and the activity was expressed as a percentage of the value obtained without added antibody. Nonimmune rabbit serum had no effect on the enzymatic activity. Preincubation with antibody did not increase the inhibition, and the presumed antibody−antigen complex was not removed from the incubation before the assay of enzymatic activity was performed. Antibody to mobilferrin (B). The monooxygenase activity was inhibited by polycolonal antibody to mobilferrin. Twenty-five microliters of enzyme (30 μg) was incubated with 10 μL of immune serum in various dilutions for 10 min at 37 °C. Then 900 μL of prewarmed buffer containing 0.25 mM NADPH and 0.6 mM Fe−NTA was added, and the change in absorbance at 340 nm was determined. The dilution of serum was expressed as the final dilution in the complete incubation mixture. The activity was expressed as a precentage of the value in the absence of immune serum. For each value, a control of the absorbance change due to the serum alone without enzyme was subtracted. Pre-immune sera did not affect oxygenase activity.

as methimazole (Williams et al., 1985; Poulsen et al., 1986). The protein was shown to contain flavin by fluorescence spectroscopy, and the flavin was identified as FAD. The mobilferrin/calreticulin component of paraferritin has been previously described as forming a tight complex with FMO (Guan et al., 1991).

Since paraferritin had a ferrireductase activity and an associated FMO, it seemed likely that the FMO functioned to reduce the iron. FMO's typically utilize one atom of

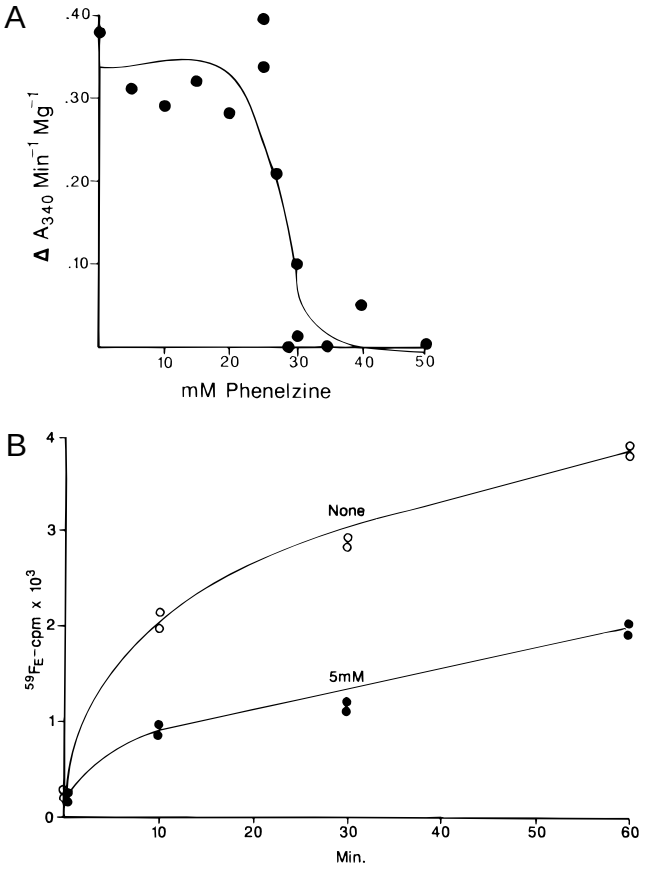

FIGURE 11: Effect of phenelzine on monooxygenase activity and iron transport. Inhibition of enzyme activity (A). The monooxygenase activity utilizing Fe−NTA as substrate was determined as described under Experimental Procedures. To the incubation were added various concentrations of phenelzine, and the change in absorbance at 340 nm was determined. Inhibition of iron transport (B). Iron uptake into IEC-6 cells in tissue culture was determined as described under Experimental Procedures, with the uptake determined in duplicate over 60 min (open circles). In additional samples, the effect of 5 mM phenelzine on iron uptake (closed circles) was determined.

oxygen to hydroxylate substrates, with the other oxygen appearing in H_2O. However, iron was not hydroxylated, but reduced. Substrates that cannot be hydroxylated by monooxygenases still oxidize NADPH, but H_2O_2 was produced (Tynes et al., 1985; Massey & Hemmerich, 1975) as was the case for paraferritin. The mechanism has still not been completely elucidated, but may involve a flavin peroxide and release of superoxide (Massey & Hemmerich, 1975). The production of hydrogen peroxide and superoxide was most consistent with a monooxygenase-like mechanism. Paraferritin did not reduce cytochrome *c* (except via superoxide), and did not have a classic flavin dehydrogenase activity. The most consistent mechanism would be for one electron to be donated by paraferritin to iron, and the second donated to oxygen, probably forming superoxide that might dismutate to hydrogen peroxide. This mechanism must still be considered tentative. Presumably, free radical formation (*Fenton reaction*) was avoided *in vivo* by the continual binding of iron by mobilferrin and the rapid removal of peroxide by peroxidases. Otherwise, the free radicals produced by the *Fenton reaction* could damage cells. Although FMO's catalyze the oxygenation of many drugs and xenobiotics, their physiologic role is controversial. The association of FMO with paraferritin suggests that it may be important in converting Fe(III) to Fe(II) *in vivo*. The velocity of the reduction of Fe(III) as determined by the rate of NADPH consumption was analogous to the rates reported for other FMO substrates (Kimura et al., 1983). Only liver microsomal FMO with thiourea (Tynes et al., 1985), cysteamine (Williams et al., 1984), or octylamines (Poulsen et al., 1986) was reported with substantially greater V_{max}. It is possible because of the presence of integrin and FMO which are usually associated with plasma membranes that the paraferritin complex originates at the plasma membrane (Imman et al., 1994).

The exact role of paraferritin in the mobilferrin−integrin pathway was not clear and is currently being investigated. Both the integrin and mobilferrin components found in paraferritin have been convincingly implicated in iron absorption (Conrad et al., 1993a). The rapid radiolabeling of paraferritin *in vivo* in pulse−chase experiments following intraluminal administration of $^{59}FeCl_3$ suggested a role for paraferritin in the mucosal uptake of iron. FMO in paraferritin provides a means to reduce ferric iron either in the surface of the cell or in the cytosol. Before iron can be utilized for certain biosynthetic reactions such as the synthesis of heme, the Fe(III) must be converted to Fe(II).

Demonstration of integrin in an iron binding water-soluble complex was not anticipated. Mobilferrin/calreticulin have previously been reported in tight association with the cytoplasmic portion of α integrin chains (Rojiani et al., 1991). Solubilization may be mediated by a mechanism similar to that suggested for FMO (Guan et al., 1991). The complex chromatographed on an Ultrogel ACA22 column with an apparent molecular mass of 520 kDa, and it did not elute in the void volume where membrane fragments would be anticipated. Essentially, no activity or protein was sedimented at 134000*g* for 1 h. That the radioiron remained bound to the complex was indicated because free radioiron would precipitate at pH 7.4 and ionic iron would avidly bind to the resins in DE-52 anion exchange columns. Intact integrins appeared to be present. Protein staining bands of an appropriate molecular mass for α subunits (150 kDa) and β subunits (90 kDa) were observed on SDS−PAGE gels, and these were identified immunologically. Further, the complex bound fibronectin, mobilferrin, and flavin monooxygenase, indicating that functions of both the *extracellular* (fibronectin binding) and *cytosolic* (mobilferrin) portions of the integrin remained intact. The solubilized integrin may, however, have been modified in some manner.

This paper does not explore the function of paraferritin except that it binds iron in a reversible fashion and can behave as a ferrireductase. The inclusion of the membrane integrin in the complex suggests it may function as a mechanism of internalizing iron for use by the cell. The presence of flavin monooxygenase suggests a mechanism whereby the complex acts as a ferrireductase. This activity is probably essential either for entry of iron into the cell or for utilization of iron by the cell or both. There may be other functions associated with paraferritin because we may not have identified all the subunits. Thus, the exact functions and activity of paraferritin require further investigation.

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