Discovery of a cytosolic/soluble ferroxidase in rodent enterocytes

Perungavur N. Ranganathan^{a,1,2}, Yan Lu^{a,1}, Brie K. Fuqua^{b,c}, and James F. Collins^{a,2}

^aDepartment of Food Science & Human Nutrition, University of Florida, Gainesville, FL 32611; ^bDepartment of Nutritional Science and Toxicology, University of California, Berkeley, CA 94720; and ^cIron Metabolism Laboratory, Queensland Institute of Medical Research, Brisbane, QLD, Australia

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Hephaestin (Heph), a membrane-bound multicopper ferroxidase (FOX) expressed in duodenal enterocytes, is required for optimal iron absorption. However, sex-linked anemia (*sla*) mice harboring a 194-amino acid deletion in the Heph protein are able to absorb dietary iron despite reduced expression and mislocalization of the mutant protein. Thus Heph may not be essential, and mice are able to compensate for the loss of its activity. The current studies were undertaken to search for undiscovered FOXs in rodent enterocytes. An experimental approach was developed to investigate intestinal FOXs in which separate membrane and cytosolic fractions were prepared and FOX activity was measured by a spectrophotometric transferrin-coupled assay. Unexpectedly, FOX activity was noted in membrane and cytosolic fractions of rat enterocytes. Different experimental approaches demonstrated that cytosolic FOX activity was not caused by contamination with membrane Heph or a method-induced artifact. Cytosolic FOX activity was abolished by SDS and heat (78 °C), suggesting protein-mediated iron oxidation, and was also sensitive to Triton X-100. Furthermore, cytosolic FOX activity increased ~30% in iron-deficient rats (compared with controls) but was unchanged in copper-deficient rats (in contrast to the reported dramatic reduction of Heph expression and activity during copper deficiency). Additional studies done in *sla*, *Heph*-knockout, and ceruloplasmin-knockout mice proved that cytosolic FOX activity could not be fully explained by Heph or ceruloplasmin. Therefore rodent enterocytes contain a previously undescribed soluble cytosolic FOX that may function in transepithelial iron transport and complement membrane-bound Heph.

intestine | ferrozine | cyto-FOX

Absorption of dietary iron occurs in the duodenal epithelium via the activity of trans-membrane iron-transport proteins coupled to reduction and oxidation reactions at the apical and basolateral surfaces of enterocytes, respectively. Iron export from enterocytes, presumed to be the rate-limiting step in body iron acquisition (1), is mediated by ferroportin 1 (Fpn1) but also requires an oxidase to convert ferrous (Fe^{2+}) iron to ferric (Fe^{3+}) iron, the transferrin (Tf)-binding form. This oxidation is mediated by a multicopper ferroxidase (FOX), hephaestin (Heph), which contains a single membrane-spanning domain and is associated with the basolateral membrane of enterocytes (2, 3). Mice harboring a major deletion in the *Heph* gene (sex-linked anemia; *sla*) express lower levels of a truncated Heph protein that is also mislocalized in intestinal epithelial cells (2). Despite this anomaly, adult *sla* mice generally display a mild iron-deficient (FeD) phenotype (4) (Table S1), suggesting that another FOX can compensate for the lack of Heph. One possibility is that a circulating multicopper FOX, ceruloplasmin (Cp), could play this compensatory role. Cp was noted in the lamina propria of mouse villi under conditions of low-iron stress and was hypothesized to facilitate iron absorption (5).

Previous investigations of enterocyte Heph expression and activity were performed in whole-cell lysates prepared in the presence of 1.5% (wt/vol) Triton X-100 (1, 4). The rationale for using this high detergent concentration, which is >70 times the critical

micelle concentration for Triton X-100, was not explained. Therefore the possibility that the activity of additional FOXs could have been masked was considered. Enterocyte lysates were prepared and separated into cytosolic/soluble and membrane/particulate fractions, and a spectrophotometric Tf-coupled FOX assay was performed. Soluble/cytosolic fractions of rat enterocytes contained notable FOX activity. This activity could be a method-induced artifact or caused by contamination of the cytosolic fraction with membrane proteins (e.g., Heph). Experiments designed to test these two possibilities eliminated any doubts. Studies were undertaken subsequently to determine the biochemical and functional properties of this FOX. Extensive additional experiments in FeD and copper-deficient (CuD) rats and in mice with mutations or deletions of known FOXs indicated that this cytosolic FOX (cyto-FOX) activity is protein-mediated and that it could not be fully explained by Heph or Cp. This FOX could complement membrane Heph and may explain, in part, the lack of a severe FeD phenotype in *sla* mice.

Results

Analysis of Experimental Animals. FeD and CuD rats were significantly anemic compared to controls (Table S1), validating the dietary regimen. Enterocyte iron content of FeD rats was reduced >90% compared with controls; mean copper levels, although notably higher in the FeD group, did not show a statistically significant increase ($P = 0.11$). Quantitative RT-PCR (qRT-PCR) analysis of isolated rat enterocytes showed increases in Menkes copper ATPase (*Atp7a*; ~9.3-fold; $P < 0.05$), copper transporter 1 (~2.3-fold; $P < 0.01$), Heph (~2.4-fold; $P < 0.01$), and metallothionein 1A (~10.2-fold; $P < 0.01$) mRNA expression in FeD rats compared with controls ($n = 8$ control and 8 FeD rats, each assayed separately). These observations provide further evidence of iron deficiency and are in agreement with previously published observations (6, 7). Furthermore, *sla* mice used for these studies were not anemic, as indicated by lack of differences in hemoglobin (Hb) and hematocrit (Hct) compared with WT mice, whereas *Cp*^{-/-} mice were mildly anemic with notable reductions in Hb and Hct (Table S1).

FOX Activity in Rat Enterocyte Fractions. To assess FOX activity in enterocytes, which previously had been measured only in total lysates prepared in the presence of 1.5% Triton X-100 (1, 4), cell lysates were fractionated to obtain cytosolic/soluble and membrane portions. FOX activity was then tested in these fractions from control and FeD rats using a coupled *apo*-Tf assay. Surprisingly, both fractions exhibited FOX activity, with the

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¹P.N.R. and Y.L. contributed equally to this work.

²To whom correspondence may be addressed. E-mail: pnr@ufl.edu or jfcollins@ufl.edu.

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Fig. 1. FOX activity in rat enterocyte fractions. Relative catalytic rate (dA₄₆₀/dt) is shown at the 1-, 2-, and 3-min time points. (A and B) Mean $\pm$ SD of data from 12 individual rats in three separate experiments. ***P* < 0.01; **P* < 0.05. Ctrl, control rats; FeD, iron-deficient rats.

enzymatic rate (dA/dt) decreasing as a function of time, reflecting substrate depletion (Fig. 1). Also notable was the increase in cyto-FOX activity in FeD rats (~30%), with no significant difference in membrane FOX activity noted between

groups (Fig. 1A). These data provide preliminary enzymatic evidence of a cytosolic FOX; subsequent experiments were designed to determine if this result was an artifact or caused by contamination with membrane-bound Heph.

Purity of Rat Enterocyte Subcellular Fractions. First, the relative purity of the fractions was assessed. The cytosolic/soluble fraction contained robust lactate dehydrogenase (LDH) activity ($\text{dA}_{340}/\text{dt}$), representing an established marker enzyme for cytosol (Fig. 2A). LDH activity was significantly higher in cytosol than in whole-cell lysate. Additional experiments were done by immunoblot analysis using antibodies against two transmembrane proteins [zinc transporter 1 (ZnT1) (Fig. 2B) and Atp7a (Fig. 4B)]. Neither protein was detected in enterocyte cytosolic fractions (even upon overexposure of the blots), but strong immunoreactive bands were detected in membrane samples, as expected.

FOX Activity in Cytosolic Fractions Prepared by Additional Methods.

Next, to eliminate potential shear-induced mechanical damage to the isolated proteins (and possible contamination of the cytosolic fraction with membrane proteins), cytosolic/soluble fractions were prepared by two complementary methods that achieved cell lysis by exposure to hypotonic solution or freeze-thaw cycles. Two individual samples from different rats were tested by each of the three lysis methods (grinding, hypotonic, and freeze-thaw). Progress curves from Tf-coupled FOX assays demonstrated that FOX activity showed similar profiles in all methods tested (Fig. 2 *C* and *D*). The congruence of data from multiple methods enhances the validity of the observation that a soluble FOX exists in enterocyte cytosol.

Inhibition Studies of Rat Enterocyte Cyto-FOX. Whether cyto-FOX activity was protein mediated and whether it was sensitive to a detergent used previously to study enterocyte Heph activity was

Fig. 2. Enterocyte purity and alternative methods. (A) LDH activity assays. Catalytic rate at 75 s is shown reflecting disappearance of the substrate pyruvate. Lysate and cytosol are samples purified from rat enterocytes ($n = 3$). Purified rabbit muscle LDH (0.05 U, 0.1 U, and 0.2 U) was used as a positive control. $^{**}P < 0.01$ compared with lysate. (B) Western blot of four rat cytosol and four membrane samples reacted with anti-ZnT1 antibody. (Lower row) Ponceau S-stained blot showing comparable loading and transfer of proteins. The black line indicates where unrelated lanes were removed from the image. (C and D) Progress curves from Tf-coupled FOX assay of enterocyte cytosolic and membrane fractions prepared using alternative methods. Each symbol is the mean of two individual rats. In A, C, and D, blanks are reaction mixes devoid of enzyme source (i.e., protein sample).

determined next. Cyto-FOX activity was abolished by treatment with 1% SDS and by heating samples to 78 °C (Fig. 3A). When 1.5% Triton X-100 was added to samples just before FOX assay, a significant reduction in enzyme activity was observed, as indicated by the divergent slopes of the progress curves (Fig. 3B).

Cyto-FOX Activity in Copper-Deficient Rats. The expression and activity of two multicopper FOXs (Heph and Cp) are reduced significantly during copper deficiency in rodent and cell culture models (8–10). A logical question was whether cyto-FOX activity was similarly responsive to dietary copper levels. A previously developed feeding regimen was used in which weanling rats were fed a low-copper diet for ~38 d (11). As expected, rats had significant reductions in Hb and Hct levels indicative of severe copper deficiency (Table S1). Moreover, serum Cp levels were reduced dramatically (Fig. 4A), and enterocyte Atp7a expression was increased significantly (Fig. 4B), consistent with previous observations made in CuD mice (12). More importantly, cyto-FOX activity was not different in the normal controls compared with the anemic CuD rats (Fig. 4C).

Analysis of Cyto-FOX Activity in Mutant Mouse Models. To further consider possible contributions of Heph or Cp to cyto-FOX activity, studies were performed in *sla*, *Heph*^{-/-} (i.e., *Heph*-KO), and *Cp*-KO mice. Immunoblot analysis showed that Heph protein was not detectable in cytosolic fractions of enterocytes isolated from *sla* mice (Fig. 5A) and that Cp protein was not detected in cytosolic fractions from enterocytes of any mice studied but was present in mouse serum (Fig. 5B). Moreover, *Heph*-KO mice contained no immunoreactive Heph protein using two antibodies (one, D4, provided by Chris Vulpe, University of California, Berkeley, CA and one obtained from GeneTex), whereas WT controls had a strong immunoreactive Heph band. Last, cyto-FOX assays demonstrated very similar enzyme activity in *sla* and *Cp*^{-/-} mice compared with WT mice (Fig. 5C and D).

Although cyto-FOX activity was modestly reduced in *Heph*-KO mice, the difference did not reach statistical significance (Fig. 6A). This observation was confirmed when FOX activity by ferrozine assay also showed no significant differences between WT controls and *Heph*-KO mice (Fig. 6B).

Discussion

This study considered the possibility that additional, unidentified FOXs exist in mammalian enterocytes. In this regard, two important questions are worth considering: (i) Is a FOX absolutely required for intestinal iron absorption, and, if so, (ii) is Heph adequate? As to the first question, the predominance of evidence suggests that enzymatic oxidation of iron is required to provide physiologic levels of iron for normal homeostasis. The chemical oxidation of Fe²⁺ to Fe³⁺ by O₂ is a complex process, influenced by pH, ionic strength, and temperature, involving many hard-to-characterize/-predict, metastable intermediates, and finally yielding not one but a variety of iron oxides (13). Even at pH 7.35 (in contrast to pH ~5.0 that was used here), under air saturation, the enzymatic rate is four times as fast as the nonenzymatic rate (14). Most important, comparing the estimated rates of Fe³⁺-Tf formation in human sera at pH 7.35 in enzymatic (300 ± 60 μM/min) and chemical (24 μM/min; ~7–10% of the former) routes, Osaki et al. (15) concluded that the chemical route is inadequate to supply sufficient iron for synthesis of biomolecules such as Hb. Furthermore, informative comparisons can be made with another redox-active metal, copper, which traverses enterocytes as Cu⁺ but does not require an oxidation step for release from the intestinal epithelium and transport as Cu²⁺ in the serum. Extrapolating the apparent adequacy of chemical oxidation for Cu⁺ to Cu²⁺ to iron reveals distinct differences, because the standard reduction potential at pH 7.0 for Fe³⁺ + e⁻ to Fe²⁺ is 0.77 V, whereas that for Cu²⁺ + e⁻ to Cu⁺ is 0.15 V (19% of the former), indicating a greater propensity for copper to be oxidized spontaneously. Enzymatic oxidation of dietary iron thus appears to be critical for maintaining normal iron homeostasis.

As to whether Heph alone is adequate to mediate iron absorption, the mutation and mislocalization of Heph in *sla* mice

Fig. 3. Chemical properties of cyto-FOX. (A) Effect of protein denaturants on enzyme activity. Data shown are reaction rates at 30 s from four rats per treatment. (–), no treatment. ****P* < 0.0001 compared to (–). (B) Effect of 1.5% Triton X-100 (TX) on cyto-FOX activity. Progress curves are shown for blank and cytosol samples with and without the addition of detergent. *n* = 4 individual rats. **P* < 0.05. In A and B, data are shown as mean ± SD.

Fig. 4. Effect of copper deficiency on cyto-FOX activity. (A and B) (Upper) Western blot of Cp protein in rat serum (A) and Western blot of Atp7a protein in duodenal enterocyte samples derived from experimental rats (B). 54–10 indicates the particular antiserum used. (Lower) Ponceau S-stained blots exemplify equal loading and transfer of protein. (C) Cyto-FOX activity assays in samples derived from experimental rats. The enzymatic rate (dA₄₆₀/dt) is shown at various time points. Data are shown as mean ± SD. *n* = 5. Ctrl, control rats; CuD, copper-deficient rats.

Fig. 5. Heph and Cp protein expression and FOX activity in mutant mouse models. WT mice, *sla* (*Heph* *sla/y*), and *Cp*-KO (*Cp*^{-/-}) mice were studied. (A and B) (Upper) Western blot for cytosolic Heph in three WT (+/y) and three mutant (*sla/y*) mice (A) and Western blot for Cp in three WT, three *sla/y*, and two *Cp*^{-/-} mice (B). MW, molecular weight markers. (Lower) Ponceau S-stained blots exemplify equal loading and transfer of protein. Black lines indicate where unrelated lanes were removed from the images. (C and D) FOX activity assays are shown as mean $\pm$ SD. Enzymatic rate (dA₄₆₀/dt) is shown at various time points. $n = 3$ WT, 6 *sla/y*, and 3 *Cp*^{-/-} mice.

does not result in a null phenotype for iron absorption, because only a mild/moderate iron deficiency is observed (16) (Table S1). Further, as a catalyst, the sole function of an enzyme is to help the reaction attain equilibrium much faster than via the uncatalyzed route, provided that Gibbs free energy (ΔG) allows the reaction to occur spontaneously. In this vital function, a K_{cat} of 2.0 estimated for Heph (17) is tantamount to an uncatalyzed reaction, whereas many other enzymes have values three to seven orders of magnitude higher. For example, catalase, belonging to the same enzyme group, has a K_{cat} of $\sim 100,000$ (18). These facts raise questions about the adequacy of Heph as the sole enterocyte FOX to mobilize needed amounts of iron, in contrast to the proposal by Hellman and Gitlin (19).

Two fortuitous alterations to previously used experimental approaches aided us in identifying an undiscovered cytosolic FOX activity in rat enterocytes: (i) Enterocyte lysates were prepared initially in the absence of detergent, and relatively pure cytosolic/soluble and membrane/particulate fractions ultimately were obtained; and (ii) a spectrophotometric, Tf-coupled FOX assay was used, allowing more precise and sensitive measurement of enzyme activity. Initially it was considered that this cyto-FOX activity could be the result of contamination of cytosolic fractions with membrane Heph or a method-induced artifact. Several notable facts argue against both these possibilities: (i) Cytosolic fractions have robust activity of a commonly used marker enzyme; (ii) immunoblotting for two membrane proteins revealed little if any immunoreactive protein in cytosolic fractions; (iii) cytosolic fractions prepared using three distinct methods, two of which eliminate the grinding step, all showed similar cyto-FOX activity; (iv) cyto-FOX activity is present in severely CuD rats, a physiologic state in which Heph expression and activity have been shown to decrease dramatically; and (v) cyto-FOX activity is not different when comparing Heph mutant (*sla*) or *Heph*-KO mice with WT controls. Thus it is unlikely that cyto-FOX activity can be fully explained by Heph, although

a possible contribution by a soluble/alternative form of Heph cannot be ruled out completely. Another possibility was that cyto-FOX could be Cp, because Cp was detected in mouse enterocytes and in the lamina propria of mouse villi (5). To consider this alternative, a well-established Cp antibody was used to probe Western blots of cytosolic proteins purified from isolated mouse enterocytes. Immunoreactive Cp was never detected in cytosolic fractions, but it was detected consistently in mouse serum.

In summary, enzymatic oxidation of iron appears essential, and Heph alone may not be adequate. The current investigation provides multiple lines of evidence supporting the existence of a unique, soluble FOX in rodent enterocytes. Of significance is the fact that this cyto-FOX activity increased during iron deficiency, whereas membrane FOX activity did not. Furthermore, the lack of a significant reduction in cyto-FOX activity in CuD rats and the presence of cyto-FOX activity in *sla*, *Heph* *-y* and *Cp*-KO mice, suggest that a previously undescribed or Heph-unrelated protein mediates this activity. This newly discovered FOX may have an important physiologic role in intestinal iron absorption, perhaps complementing the function of membrane-bound Heph.

Methods

Chemicals and Reagents. Chemicals were purchased from Sigma Aldrich and Fisher Scientific and were of analytical grade or high purity. Other sources are mentioned as appropriate.

Animals, Diets, and Tissue Collection. All rat studies were approved by the University of Florida Institutional Animal Care and Use Committee. Weanling male Sprague-Dawley rats (Harlan) were raised in our animal facility according to standard procedures (11) in overhanging, wire mesh-bottomed cages and were fed one of three AIN-93G-based diets (Dyets, Inc.) with the following iron and copper concentrations: control, ~ 200 ppm iron, 6 ppm copper; FeD, ~ 3 ppm iron, 6 ppm copper; and CuD, ~ 200 ppm iron, <1 ppm copper. Diets were otherwise identical. After 35–42 d on the diets, rats were killed by CO₂ exposure followed by cervical dislocation. Blood was collected

Fig. 6. FOX activity in enterocyte cytosol from *Heph*-KO mice. FOX activity was measured using two spectrophotometric methods, the initial velocity Tf assay (A) and the end-point ferrozine (Fz) assay (B). The enzymatic rate (dA/dt) is shown at different time points for the Tf assay. A_{570} progress curves are shown for the ferrozine assay. Note that the ferrozine assay is a substrate disappearance assay in which lower numbers indicate higher enzyme activity. No statistical differences were noted between groups in either assay. $n = 4$ for WT and 4 for *Heph* $-/-$ mice.

by cardiac puncture. Hb and Hct were measured by standard methods (11). *Sla*, *Heph* $-/-$, and *Cp*-KO mice were bred at the animal facility of the University of California, Berkeley following guidelines approved by the Office of Laboratory Animal Care. *Sla* mice ($n = 6$) were 8–10 mo old, male *Cp*-KO mice ($n = 3$) were 12–17 mo old, and male WT (C57BL/6J) mice ($n = 7$) were ~1.5 to 7 mo old. *Heph*-KO mice (developed and characterized by Gregory J. Anderson, Queensland Institute of Medical Research, Brisbane Australia and Chris Vulpe, University of California, Berkeley, CA) were also used. WT ($n = 4$) and *Heph*-KO ($n = 4$) mice were sexually mature 17- to 34-wk-old males. Note that *sia*, *Heph*-KO, and *Cp*-KO mice were maintained on the C57BL/6J genetic background.

Elemental Analysis. Frozen enterocytes were submitted to the Diagnostic Center for Population and Animal Health at Michigan State University for mineral analysis. Briefly, samples were dried overnight at 95 °C, digested with nitric acid, and then subjected to inductively coupled plasma-mass spectrometry analysis.

Enterocyte Isolation. Enterocytes were isolated from duodenum and proximal jejunum of rats and mice as described (20). Approximately 15 cm of rat proximal intestine (~12 cm for mice) was removed immediately after death and flushed with ice-cold PBS, cut into ~5-cm lengths (~4 cm for mice), and everted on individual wooden sticks. After two brief washes in PBS, gut segments were incubated in ice-cold PBS containing 1.5 mM EDTA plus protease inhibitors for 20 min (15 min for mice) with gentle rotation to release enterocytes, which subsequently were pelleted at 500 $\times g$ for 5 min. Enterocytes were washed three times with PBS and used immediately for fractionation, and fresh samples were used for FOX assay and immunoblot analysis. Enterocytes were frozen at -20 °C for qRT-PCR and mineral analysis.

Subcellular Fractionation. Method I (grinding). Cytosolic and solubilized particulate/membrane fractions were prepared as described (21). All steps were performed at 4 °C. Briefly, enterocytes were homogenized by a tissue grinder in buffer 1 [0.025 M Tris-HCl (pH 7.4), 0.025 M NaCl, plus protease inhibitor mixture: 1 μ g/mL pepstatin A, 100 μ M leupeptin, 4 mM benzamide, and 1

mM PMSE] and centrifuged at 16,000 $\times g$ for 15 min. The supernatants were recentrifuged at 110,000 $\times g$ for 1 h. The resulting supernatants were termed the “cytosol” fraction. The pellets were resuspended in buffer 2 [buffer 1 with 0.25% (vol/vol) Tween-20], sonicated 2 $\times$ 5 s at 5 W in an ice-water slurry with 15-s chilling between sonications, and recentrifuged at 16,000 $\times g$ for 30 min. These supernatants were termed the “membrane” fraction.

Method II (hypotonic lysis). Enterocytes were incubated in buffer 1 on ice for 30 min with frequent mixing with 1-mL pipette tips and centrifuged at 16,000 $\times g$ for 15 min. Subsequent steps were identical to method I.

Method III (freeze/thaw). Enterocytes were suspended in buffer 1, snap-frozen in liquid nitrogen, and then quickly thawed in a 37 °C water bath. This temperature-change cycle was repeated five times, and lysis of cells was confirmed visually under a microscope. Subsequent steps were identical to method I. Protein concentrations were estimated by Pierce 660-nm protein assay (Thermo Fisher Scientific).

qRT-PCR. Quantification of enterocyte mRNA expression was performed by standard methods using SYBR Green and validated gene-specific primers, as described previously (11). Primer sequences are listed in Table S2.

Western Blot Analysis. Thirty micrograms of cytosolic or membrane proteins were resolved by SDS-7.5% PAGE, blotted, and immunoprobed as described earlier (21). The anti-Atp7a antibody (called “54-10”) (20, 22) and anti-ZnT1 (a kind gift from Robert Cousins, University of Florida, Gainesville, FL) (23) are well validated. Rabbit polyclonal anti-Heph Ab was from GeneTex (cat. # GTX115300), and chicken polyclonal anti-Cp Ab was a kind gift from Sigma (cat. # GW20074F). Goat anti-rabbit HRP-conjugated secondary antibody was from Bethyl Laboratories (cat. # A120-101P), and anti-chicken HRP-conjugated secondary Ab was a kind gift from Sigma (cat. # A9046). Immunoreactive bands on blots were visualized by enhanced chemiluminescence following a standard protocol (11). Ponceau S-stained blots were used to confirm comparable sample loading and transfer.

Enzyme-Activity Assays. All enzyme-activity assays except the ferrozine assay were performed at room temperature using a Beckman DU 640 spectrophotometer, running parallel blanks containing complete reaction mix minus sample protein.

LDH initial velocity assay. The reaction mix for the LDH assay contained 0.002 M sodium pyruvate in 0.15 M Tris-HCl buffer (pH 7.2) with or without sample protein. Reaction was initiated by adding NADH to 200 μ M in a total volume of 200 μ L. Initial velocities for 75 s were obtained by following the dA_{340}/dt . Rabbit muscle LDH (Sigma) was used as a positive control.

Tf-coupled FOX initial velocity assay. In the Tf-coupled FOX assay, FOX catalyzes the oxidation of Fe^{2+} to Fe^{3+} , which subsequently binds to apo-Tf, resulting in an Fe^{3+} -Tf complex with an A_{max} at 460 nm. The reaction mix contained equal amounts (60 μ g) of cytosolic or membrane protein from enterocytes in 0.125 M CH_3COONa - CH_3COOH buffer, pH 5.0, and 50 μ M bovine apo-Tf (Sigma). Reaction was initiated by adding $(NH_4)_2Fe(SO_4)_2$ to 50 μ M in a final reaction volume of 200 μ L. Initial velocities from 15 s to 3 min were obtained by following the dA_{460}/dt .

Inhibition studies. For inhibition studies, cytosolic fractions from four individual control rats were used. One percent SDS (final concentration) was pre-incubated with the lysate at room temperature, or the lysate was heated to 78 °C for 20 min before the addition of Fe^{2+} and apo-Tf.

Ferrozine FOX end-point assay. The ferrozine assay was performed following an established procedure (11). Briefly, 1.0 mL reaction mix containing 0.125 M CH_3COONa buffer (pH 7.0), 50 μ M $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$, plus equal quantities of enterocyte cytosolic protein (60 μ g) from each animal was mixed and incubated at 37 °C. Aliquots were drawn at indicated time points, the reaction was terminated with 3 mM ferrozine, and A_{570} was measured indicating levels of ferrous iron remaining in the sample.

Statistical Analysis. Results were expressed as mean $\pm$ SD when $n \geq 3$. The data were analyzed by Student’s *t* test for comparisons between two treatment groups using Microsoft Office Excel software and Prism GraphPad (version 4.0c). $P < 0.05$ was considered statistically significant.

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