Supporting information for Barnés *et al.* (2002) *Proc. Natl. Acad. Sci. USA* **99** (8), 5195–5200. (10.1073/pnas.032089399)

Supporting Text

Experimental Methods

General. All starting materials were purchased from Sigma, Aldrich, Biorad, or Strem Chemicals and were used without further purification, unless otherwise specified. The water used for the experiments was purified to 18 MΩ by using a reverse osmosis-charcoal demineralizer system (Millipore). Apoferritin concentrations were determined by the Bradford method by using BSA as a standard (1), and confirmed spectrophotometrically by using a molar absorptivity of $\in_{280nm} = 4.8 \times 10^5\ M^{-1}cm^{-1}$ per ferritin. $^{51}CrCl_3$ was obtained from New England Nuclear; ^{51}Cr samples were counted on a TRI-CARB 2100-TR Scintillation Counter (Packard). Elemental analyses were performed at the Microanalysis Facility in the University of California at Berkeley. For chromium analyses, samples were digested in a 1:1 volume with concentrated nitric acid and boiled for 1 h. Electronic absorption spectra were recorded on a Hewlett–Packard HP8452A instrument by using 1-cm path-length quartz cells. Stopped-flow UV-vis data were collected on an Applied Photophysics instrument (Surrey, U.K.).

Synthesis of Cr(TREN). $[Cr(TREN)Cl_2]Cl$, (0.6 g, 2 mmol), prepared according to published procedures (2, 3), was combined with anhydrous CF_3SO_3H (15 ml) and stirred overnight at room temperature under N_2. The product, $[Cr(TREN)(CF_3SO_3)_2]–[(CF_3SO_3)]$, was precipitated by a dropwise addition of diethyl ether until the supernatant was almost colorless (light pink). The pale precipitate was then removed by filtration under a nitrogen flow, washed with diethyl ether, and dried under vacuum. Anal. calcd. (found) for $Cr_1C_9H_{18}N_4F_9O_9S_3$: C, 16.75(16.67); H, 2.81(2.85); N, 8.68(8.40). UV-vis [5.6 M CF_3SO_3H, λ_{max} (nm); $\in$ (M^{-1} cm^{-1})]: (assignments for an O_h ligand field) $^4A_{2g} \rightarrow {}^4T_{2g}$ 502 (97.8), and $^4A_{2g} \rightarrow {}^4T_{1g}$ 378 (51.7). To synthesize the radiolabeled compound, $^{51}CrCl_3$ was mixed with unlabeled $CrCl_3$ to yield an activity of 1 mCi/mmol (1 Ci = 37

GBq), and the complex was prepared as described above. Cr(TREN) stock solutions were prepared by dissolving the compound $[Cr(TREN)(OSO_2CF_3)_2][(CF_3SO_3)]$ in acidic water (pH 3.0) to the desired concentration (1 to 10 mM); solutions were kept frozen until use. Chromium concentrations were confirmed by UV-vis and chromium elemental analysis by inductively coupled plasma atomic emission spectrometry (ICP).

Preparation of Ferritin and Cr(TREN) Binding. Most ferritins are composed of H subunits with ferroxidase sites. However, in vertebrates L subunits, with inactive ferroxidase sites, associate with H subunits in ratios that are genetically controlled by cell type. Four recombinant ferritins and one natural tissue ferritin were compared (Table 1).

Recombinant proteins used were frog sequences isolated from plasmids in *Escherichia coli* as assembled, demineralized proteins: rH, wild type sequence (56); rH, L134P-localized unfolding at the threefold pore (20); rL, wild-type sequence (57); rL-E2, rL ferritin with substitution of four conserved Glu by Ala at the twofold axis nucleation site (3). HoSF is commercial horse spleen ferritin with iron removed by reduction with thioglycolic acid (58); n.d., not determined. Binding was measured by equilibrium dialysis (Fig. 3), Fe^{2+} oxidation as the change in absorbance at 350 nm (Fig. 4), and Fe^{3+} mineralization as the density of Prussian blue formed when solutions of Cr(TREN)-protein complexes were mineralized with solutions of $FeSO_4$ (480 Fe/ferritin) and fractionated by electrophoresis in native polyacrylamide gels followed by immersion of the gels in acidic solutions of ferrocyanide after (Fig. 5).

The recombinant ferritins, used here to permit comparisons of Cr(TREN) interactions for each subunit type, were from sequences expressed in *Escherichia coli* and purified as previously described for L-subunit-type ferritin: (rL) (4, 5), L ferritin containing four mutations at the nucleation/chelation site residues, rL-E2 (Glu 56 → Ala, Glu 57 → Ala, Glu-58 → Ala, and Glu-60 → Ala) (6), H ferritin (rH), and H ferritin containing the mutation Leu-134 → Pro, rH-L134 (7, 8). In addition, the effect of mixed subunits in chromium binding was studied in horse spleen ferritin, (≈84% L). Horse spleen apoferritin (HoSF) was purchased from Sigma and used without any further

purification. The homogeneity of HoSF H- and L-subunit-type ferritins were determined by electrophoresis in denaturing (SDS, 10% polyacrylamide) and nondenaturing (6% polyacrylamide) gels. The recombinant H- and L-ferritins as isolated contained less than 1 Fe atom per ferritin molecule, as determined by *o*-phenanthroline-Fe(II) assay after protein denaturation (9). All apoferritins were prepared in 0.1 M Mops (4-morpholinepropanesulfonic acid) buffer (pH 7.0)/0.2 M NaCl. All Fe(II) solutions were freshly prepared from $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ in 1 mM HCl. Ferritin mineralization occurred by the addition of Fe(II) in increments of 500 Fe per protein in 6–12-h intervals at 25°C. After the last addition, proteins were incubated overnight at 4°C. HoSF containing 2,000 Fe per protein was dialyzed against two changes of buffer at 4°C after the addition of all iron. For Cr(TREN) binding and inhibition experiments, protein samples were incubated for 24 h at 25°C with increasing concentration of Cr(TREN) (up to 960 Cr per protein, or 40 Cr per subunit). A typical experiment used 2.1 μM protein solution and 0 to 2.5 mM Cr(TREN) solution.

Substitution of Cr(TREN) Ligands with Protein Residues: UV-vis Spectroscopy. Binding of Cr(TREN) to both HoSF and rH-L134P was monitored by UV-vis. Cr(TREN) was added to ferritin (4.2 or 8.4 μM) to a final concentration of 48 equivalents per protein. The change in absorbance on Cr(TREN) addition was followed at 20-min intervals for 15 h at 25°C. The protein absorbance prior to Cr(TREN) addition was used as a reference. The absorbance changes of Cr(TREN) in protein-free buffered solutions were measured as a control.

Determination of Acidity Constants: Potentiometric Titrations of Cr(TREN). $[Cr(TREN)(OSO_2CF_3)_2](CF_3SO_3)$ (0.5 mM) was dissolved in 0.1 M KCl (50 ml) at 25°C under argon, and the solution was pre-equilibrated for 1 h to ensure full exchange of the trifluoromethanesulfonate ligands for water molecules. The acidity constants for the two water molecules coordinated to $[Cr(TREN)(H_2O)_2]^{3+}$ were determined by potentiometric titrations as described (10). Titrations were performed in triplicate and refined by using the program BETA90 (11). The thermodynamic reversibility of each titration was determined by cycling the titration from low to high pH and back to low pH.

Protein Mobility in Nondenaturing Gels. Mobility changes due to Cr(TREN) binding to ferritin were examined in Cr(TREN)-modified rH, rH-L134P, rL, rL-E2, and HoSF, in native gels (6%). Cr(TREN) binding to rH-L134P and HoSF was also tested in isoelectric focusing gels (pH range of 3–9 and 5–8). A linear correlation was obtained between the number of equivalents of Cr(TREN) bound per subunit (as determined by elemental analyses of Cr) versus the change in mobility in native polyacrylamide gels (R_f), and the change in isoelectric focusing point. The R_f values for mobility changes were calculated as $(Y_i - Y_o)/(Y_{top} - Y_o)$; where Y_o, Y_i, and Y_{top} are the coordinates of ferritin without Cr(TREN), Cr(TREN)-modified ferritin, and the start of the separating gel. Denaturing gel electrophoresis was performed on a Bio-Rad Mini-Protean II System, and isoelectric focusing gel electrophoresis was performed on a PhastSystem (Pharmacia). Proteins were visualized with Coomassie Brilliant Blue R-250 (Bio-Rad) and iron cores by forming Prussian blue with acidic solutions of potassium ferrocyanide (3 g/100 ml of 0.1 M HCl). The relative iron or protein concentration in ferritin, as well as their relative position on the gels, were determined by densitometry and processing the data using the public domain NIH IMAGE program (NIH IMAGE software was developed at the National Institutes of Health and is available on the on-line at http://rsb.info.nih.gov/nih-image/).

Determination of Stoichiometries of Cr(TREN) Binding to Apo- and Iron-Loaded Ferritins After Dialysis. The Cr(TREN)-modified apoferritins described in Table 1 (HoSF, rL, E2, rM, and rH-L134P) and Cr(TREN)-modified reconstituted ferritins containing 500–2,000 Fe per protein (HoSF and rH-L134P) (2.1 μM) were incubated with increasing concentrations of Cr(TREN) (0–30 equivalents per subunit) at 25°C for 24 h. Because of its lower solubility, rH (2.1 μM) was incubated with up to 40 Cr(TREN) per subunit at 37°C (rather than 25°C) for 24 h. Protein concentrations were measured after dialysis at 4°C for 16–24 h. Samples were digested in a 1:1 volume of concentrated nitric acid at 80–95°C for 1 h, and chromium concentrations were determined by ICP spectrometry. The equivalents of Cr(TREN) binding per subunit were calculated from the Cr to protein ratio, as the average of three independent measurements. The concentration of free Cr(TREN) (before dialysis) was calculated assuming that the concentrations of

bound Cr(TREN) measured after dialysis at 4°C closely resembled the concentrations of bound Cr(TREN) at equilibrium.

Determination of the Equilibrium Dissociation Constant of Cr(TREN) with Ferritin. Ferritins contain multiple binding sites per subunit, which function in iron recognition, iron transit, iron oxidation, or iron mineralization. The Cr(TREN) complex may potentially bind to any of these sites. The number of binding sites, *n*, and the equilibrium constants, K_d, were determined by nonlinear regression assuming equal and independent sites according to Eq. **1**.

$$r = \frac{n[Cr]_f}{K_d + [Cr]_f} \quad \textbf{[1]}$$

$$r = \frac{n_1[Cr]_f}{K_{d1} + [Cr]_f} + \frac{n_2[Cr]_f}{K_{d2} + [Cr]_f} \quad \textbf{[2]}$$

$$R = \sqrt{m\Sigma(r_o - r_c)^2} / \Sigma r_0 \quad \textbf{[3]}$$

Here *r* corresponds to the fraction of $[Cr(TREN)]_{bound}$ per subunit, and $[Cr]_f$ corresponds the concentration of free [Cr(TREN)] during equilibration. A two-site model as shown in Eq. **2** was also tested (12) in which n_1 and n_2 are the number of sites per subunit and K_{d1} and K_{d2} are the apparent dissociation constants. Although the values for n_1 and n_2 were held fixed during fitting, these values were also optimized by systematically varying the initial guesses for n_1 and n_2 and comparing the calculated *r* values (r_c) to the experimentally determined *r* values (r_o) according to Eq. **3** (12). *R* in Eq. **3** corresponds to the normalized rms of the difference between the observed and calculated data, r_o and r_c are the observed and calculated equivalents of Cr(TREN) bound per subunit, and *m* is the total number of data points.

The equilibrium dissociation constant of Cr(TREN) with HoSF, K_d, was determined by equilibrium dialysis (13). Equal concentrations of ^{51}Cr(TREN) were added to a two-sided chamber separated by a dialysis membrane. Protein solutions were added to one side of the chamber, and buffer was added to the other. Samples were incubated

for 24 h at 25ºC, and the activity of ^{51}Cr was measured in both the protein compartments. Equilibrium concentrations were also determined by incubating ferritins with Cr(TREN), using increasing Cr(TREN) concentrations (24 h, 24ºC), removing unreacted Cr(TREN) with an ion exchange column (Chelex), and measuring the concentration of bound ^{51}Cr. The measured concentration of bound ^{51}Cr was corrected for a fraction of unbound Cr(TREN) that was not removed by the resin. The fraction was determined from the activity remaining after passage on the ion exchange column of protein-free buffered solutions containing ^{51}Cr(TREN). Both methods yielded the same results.

Determination of Rate of Dissociation of Cr(TREN) from HoSF, k_{rev}. The dissociation rate constant was calculated directly by measuring the loss of ^{51}Cr(TREN) from equilibrated ^{51}Cr(TREN)–HoSF complexes as a function of time. HoSF (2 μM) was incubated with ^{51}Cr(TREN) at four different concentrations (0.1, 0.15, 0.3, and 1.0 mM) in an equilibrium dialysis cell at 25°C. Concentrations of bound ^{51}Cr(TREN) were determined after 24 h as described above. To measure the dissociation rate, equilibrated protein samples were dialyzed against a large volume, aliquots of the protein solutions were removed at time intervals, and their protein and ^{51}Cr concentrations were measured. During dissociation, dialysis reduces the concentration of free Cr(TREN) $\approx 10^8$-fold; consequently the dissociation of Cr(TREN) from the Cr(TREN)--ferritin complex is an irreversible reaction that was found to correspond to a simple unimolecular first-order rate law.

Inhibition of Fe(II) Oxidation. The rapid burst of oxidation in Cr(TREN)-modified H ferritins (rH and rH-L134P) was examined by stopped-flow spectrometry as described for the apoprotein (4). Unbound Cr(TREN) (excess reagent that that did not react) was removed in all samples by dialysis as described above. Cr(TREN)-modified ferritins (1.6 μM) were mixed in a 1:1 ratio with an Fe^{2+} solution, and iron oxidation was followed during the first 100 s. Data were collected on diode array detection mode at logarithmic time increments. Single wavelength spectra (350 nm and either 550 nm for rH L134P or 650 nm for rH, respectively) were extracted from the raw data and analyzed by the method of initial rates.

In addition, Fe^{2+} oxidation in HoSF and rH-L134P was monitored spectrophotometrically at longer time intervals with a Hewlett–Packard (HP 8453 and HP 8452) diode array spectrophotometer by following the formation of Fe^{3+}-oxo hydroxo species (350 nm) and Fe(III)-dimer intermediate (rH-L134P only, 550 nm) as a function of time. Cr(TREN)--protein solutions were examined after equilibration [in the presence of unbound Cr(TREN)] and after dialysis [Cr(TREN) bound]. Reaction conditions were kept constant: *T*, 25°C; pH 7.0 (0.1 M Mops); *I*, 0.2 M (NaCl). Iron uptake and mineralization was initiated by the addition of Fe^{2+} ammonium sulfate (pH 3.0) to either Cr(TREN)-modified HoSF or Cr(TREN)-modified rH L134P (2.1 μM) with a manual mixing time of 10 s.

Inhibition of Mineral Core Formation in Ferritins. Inhibition of mineralization was examined by native gel electrophoresis by monitoring the decrease in iron core formation as a function of Cr(TREN) binding. Fe(II) was added to protein samples pre-equilibrated with Cr(TREN) to a final concentration of 1 mM [500 Fe(II) equivalents per protein], and the samples were incubated for 2 h at room temperature to allow iron oxidation and mineralization to occur. Ferritin samples were examined on native polyacrylamide gels, and protein and iron bands were stained and quantified as described above. Mineralization was also studied in samples in which unbound Cr(TREN) was removed by dialysis; however, the data from studies in the absence and presence of unbound Cr(TREN) were averaged, because the variations in mineralization were within experimental error.

References

1. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.

2. Dixon, N. E., Lawrance, G. A., Lay, P. A. & Sargeson, A. M. (1984) *Inorg. Chem.* **23,** 2940–2947.

3. Zipp, S. G. & Madan, S. K. (1976) *Inorg. Chem.* **15,** 587–593.

4. Waldo, G. S. & Theil, E. C. (1993) *Biochemistry* **32,** 13262–13269.

5. Trikha, J., Waldo, G. S., Lewandowski, F. A., Theil, E. C., Weber, P. C. & Allewell, N. M. (1994) *Proteins: Structure, Function, and Genetics* **18,** 107–118.

6. Trikha, J., Theil, E. C. & Allewell, N. M. (1995) *J. Mol. Biol.* **248,** 948–967.

7. Takagi, H., Shi, D., Ha, Y., Allewell, N. M. & Theil, E. C. (1998) *J. Biol. Chem.* **273,** 18685–18688.

8. Ha, Y., Shi, D., Small, G. W., Theil, E. C. & Allewell, N. M. (1999) *J. Biol. Inorg. Chem.* **2,** 243–256.

9. Rohrer, J. S., Frankel, R. B., Papaefthymiou, G. C. & Theil, E. C. (1989) *Inorg. Chem.* **28,** 3393–3395.

10. Turowski, P. N., Rodgers, S. J., Scarrow, R. C. & Raymond, K. N. (1988) *Inorg. Chem.* **27,** 474.

11. Franczyk, T. S. (1991) Ph.D. dissertation (University of California, Berkeley), pp. 77–78 and 112.

12. Macara, I. G., Hoy, T. G. & Harrison, P. M. (1973) *Biochem. J.* **135,** 785–789.

13. Freifelder, D. (1982) *Physical Biochemistry* (Freeman, New York).