Evidence for the Participation of Intestinal Xanthine Oxidase in the Mucosal Processing of Iron†

Richard W. Topham,* Mark C. Walker, Melissa P. Calisch, and R. Wayne Williams

ABSTRACT: The intestinal source of ferroxidase activity has been purified and identified as xanthine oxidase [Topham, R. W., Woodruff, J. H., & Walker, M. C. (1981) *Biochemistry 20*, 319–324]. The dietary administration of tungsten to rats results in rapid and specific inactivation of xanthine oxidase in intestinal mucosa [Johnson, J. L., Rajagopalan, K. V., & Cohen, H. J. (1974) *J. Biol. Chem. 248*, 859–866]. Thus, tungsten-treated rats provided a convenient mechanism for studying a possible role for xanthine oxidase in the mucosal processing of dietary iron. The specific ferroxidase activity, the absorption of a test dose of ^{59}Fe, and the distribution of ^{59}Fe in mucosal cytosol were compared in control, low-iron, and tungsten-treated rats. The mucosal cytosol of tungsten-treated rats had little ferroxidase activity and showed greatly increased retention of ^{59}Fe and greatly decreased transport of ^{59}Fe to blood as compared to control and low-iron rats. Gel filtration of the mucosal cytosol obtained from each group of animals demonstrated that tungsten-treated animals had more ^{59}Fe bound to mucosal ferritin and much less ^{59}Fe bound to mucosal transferrin than control or low-iron animals. Electrophoretic studies demonstrated that this alteration in the distribution of ^{59}Fe in the mucosal cytosol of tungsten-treated rats could not simply result from changes in the relative size of the mucosal ferritin and mucosal transferrin compartments. A time course study of the effect of tungsten indicated that the change in the distribution of ^{59}Fe correlated with the loss of the ferroxidase activity of intestinal xanthine oxidase. These studies support the proposal that intestinal xanthine oxidase, by promoting the oxidation and incorporation of iron into mucosal transferrin, could play a role in the processing of dietary ionic iron in the mucosal cell.

Extensive studies from a number of laboratories have demonstrated that ceruloplasmin facilitates the mobilization of iron from tissue stores (Osaki & Johnson, 1969; Ragan et al., 1969; Osaki et al., 1970, 1971; Roeser et al., 1970; Evans & Abraham, 1973; Williams et al., 1974; Topham et al., 1980). Considerable evidence suggests that ceruloplasmin facilitates iron mobilization by promoting the oxidation and incorporation of iron into plasma transferrin (Osaki et al., 1966, 1970, 1971; Osaki, 1966; Osaki & Johnson, 1969; Roeser et al., 1970; Topham et al., 1980). Although ceruloplasmin facilitates the release of iron from tissue stores to the blood stream, it appears to play no role in the uptake of iron by the intestinal mucosal cell, the processing of iron within the mucosal cell, or the regulation of intestinal iron absorption (Lee et al., 1968; Ragan et al., 1969; Brittin & Chee, 1969; Roeser et al., 1970). A non-ferritin iron-binding protein which has properties very similar to, though not identical with, plasma transferrin has been isolated and purified from intestinal mucosal homogenates (Huebers et al., 1971, 1976; Worwood & Jacobs, 1971; Halliday et al., 1976; Valberg et al., 1977). Numerous studies suggest that this protein, designated mucosal transferrin, mediates the transcellular transport of iron in the mucosal cell (Huebers et al., 1971; Pollack et al., 1972; Halliday et al., 1976; El-Shobaki & Rummel, 1977; Savin & Cook, 1980).

A non-ceruloplasmin enzyme, which promotes the oxidation and incorporation of iron into transferrin, has been isolated and purified from intestinal mucosal homogenates and has been identified as intestinal xanthine oxidase (Topham, 1978; Topham et al., 1981). It has been postulated that intestinal xanthine oxidase, by promoting the oxidation and incorporation of iron into mucosal transferrin, could peform a function in iron absorption similar to the role ceruloplasmin serves in the mobilization of iron from tissue stores. The present paper describes in vivo and in vitro evidence which suggests that intestinal xanthine oxidase is involved in the mucosal processing of dietary iron.

Experimental Procedures

Animals and Diets. In the comparative studies of control, low-iron, and tungsten-treated animals, female Fischer rats (CDF strain) were purchased at 4 weeks of age from Charles River Laboratories, Wilmington, MA. These animals were divided into three groups and maintained on their respective diet for 10 weeks. The control group received a diet of normal iron content, the second group received a diet of low-iron content (control diet less the ferrous ammonium citrate of the salt mixture), and the third group received a tungsten-supplemented diet (control diet plus 0.7 g of sodium tungstate/kg of diet). All the diets used in these studies were prepared by the Nutritional Biochemical Division, ICN Pharmaceuticals, Inc., Cleveland, OH, and were identical in composition (purified rat diet no. 904616) except for iron deletion or tungsten supplementation.

In the time course study with the tungsten-supplemented diet, 250-g adult female Fischer rats (CDF strain) were used. These rats were initially placed on the control diet for 1 week; then, control animals were sacrificed, and the remaining animals were placed on the tungsten-supplemented diet. Additional animals were sacrificed after 1.5 and 3 days on the tungsten-supplemented diet. The remaining animals were returned to the control diet and sacrificed after 2 days on the control diet.

Apotransferrin and Apoferritin. For ferroxidase activity assays, a 2.0% (w/v) solution of iron-free transferrin (apotransferrin; Calbiochem-Behring, La Jolla, CA) was prepared in deionized, glass-distilled water and extensively dialyzed as previously recommended (Johnson et al., 1970). The apotransferrin was 98–99% pure as determined by polyacrylamide

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gel electrophoresis. Dilutions of this solution were used as the transferrin markers in the electrophoresis and gel-filtration studies.

A solution of chromatographically purified apoferritin (50 mg of protein/mL; Sigma Chemical Co., St. Louis, MO) was diluted with 0.15 M NaCl as specifically indicated in figure legends and used as the ferritin marker in electrophoretic and gel-filtration studies and also used in the vitro competition studies. This preparation was >95% pure as determined by polyacrylamide gel electrophoresis.

Administration of ^{59}Fe, Removal and Analysis of Blood, and Preparation of Intestinal Mucosa. In the comparative studies of control, low-iron, and tungsten-supplemented animals, after 10 weeks on their respective diet, the rats in each of the three groups received tracer amounts of ^{59}Fe intragastrically. Specifically, food was removed 18 h before dosing, and each rat received 4 μCi of $^{59}FeSO_4$ (200 ng) per 150 g body weight in 1 mL of 0.005 M HCl delivered by a syringe fitted with a gastric tube, the dose being checked by counting of a sample of the ^{59}Fe dose solution. Blood samples were removed from at least six rats in each diet group by cardiac puncture at 10 and 30 min after administration of the radioactive iron. Serum was prepared by centrifugation, and an aliquot of the serum was counted for radioactivity. Immediately following the removal of the blood samples, the rats in each diet group were sacrificed, and the small intestine from the gastric pylorus to the ileocecal valve was excised and separated from adhering pancreatic tissue. The gut was flushed with cold saline, blotted to remove excess fluid, placed on a stainless-steel tray, and slit open, and mucosa was removed by gently scraping with a glass microscope slide. The mucosa samples obtained from the individual animals of each diet group at each time interval after ^{59}Fe dosing were pooled, and a 20% (w/v) homogenate of each pooled mucosa sample was prepared with 0.05 M *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer, pH 7.5. Mucosal cytosol was prepared from each homogenate by centrifugation at 105000*g* for 1 h to remove unbroken cells, cell debris, mitochondria, and microsomal material. The mucosal transferrin and mucosal ferritin are almost exclusively contained in the supernatant after such centrifugation (Huebers et al., 1976) as is the intestinal ferroxidase (Topham, 1978).

An aliquot of each mucosal cytosol was analyzed for radioactive iron by liquid scintillation counting of the β^- emission of ^{59}Fe on a Beckman Model LS-100 scintillation counter. Each mucosal cytosol was also analyzed for ferroxidase activity and protein concentration, and the mucosal transferrin and mucosal ferritin were separated and analyzed for ^{59}Fe radioactivity as described in subsequent sections.

Assay of the Ferroxidase Activity and Protein Concentration of Mucosal Cytosols. The ferroxidase activity of each mucosal cytosol was measured spectrophotometrically by monitoring Fe(III)–transferrin formation as the absorbance change at 460 nm where Fe(III)–transferrin has its maximal absorption [$\Delta\epsilon_{460nm} = 2500$ cm^{-1} [Fe(III)–transferrin]$^{-1}$]. This spectrophotometric assay has been described in detail and validated in numerous previous reports (Johnson et al., 1967; Osaki et al., 1966, 1971; Osaki & Johnson, 1969; Topham & Frieden, 1970; Topham & Johnson, 1974). The application of this method specifically for the assay of the ferroxidase activity of intestinal xanthine oxidase in crude mucosal homogenates and in mucosal cytosol has also been recently reported (Topham, 1978; Topham et al., 1981).

The protein concentrations of the mucosal cytosol preparations were determined by utilizing the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA) which is based on the protein dye binding principle described by Bradford (1976).

Separation and Analysis of Mucosal Transferrin and Mucosal Ferritin from Mucosal Cytosol Preparations. In previous studies, mucosal transferrin and mucosal ferritin have been effectively separated from one another by gel filtration of mucosal cytosol preparations (Huebers, 1975; Pollack & Laskey, 1975; Huebers et al., 1976; Halliday et al., 1976; El-Shobaki & Rummel, 1977). In the present studies, columns of Sephadex G-200 (2.6 × 60 cm) were used to resolve the mucosal transferrin and mucosal ferritin from 10-mL samples of the mucosal cytosol preparations.

The elution buffer was 0.05 M Hepes, pH 7.5. The elution positions of ferritin and transferrin were determined by passing pure samples of these proteins through the gel-filtration columns. All fractions eluted from the column were individually analyzed for radioactive iron content and elution profiles of ^{59}Fe constructed.

Polyacrylamide Gel Electrophoreses. Prepolymerized 7.5% polyacrylamide separating gels (Isophore gels, Isolab, Akron, OH) were equilibrated overnight at 30 V with 0.188 M Tris–glycine buffer, pH 8.9. A 50-μL aliquot of Biotracking dye (Bio-Rad Laboratories, Richmond, CA) containing 30% sucrose was added to 200 μL of each of the mucosal cytosol preparations. Samples of the resulting preparations (25 μL, 180 μg of protein) were applied to the top of 7.5% polyacrylamide separating gels. These samples were run into the separating gels at 100 V for 15 min. The electrophoretic runs were completed at 150 V for 3.5 h. The gels were fixed with a solution of 10% trichloroacetic acid, stained with Coomassie blue, and destained with 7% acetic acid. The stained gels were scanned for protein at 620 nm with an ISCO Model 1310 gel-scanning attachment for the ISCO Model UA-5 absorbance monitor.

Results

Effect of Tungsten on the Ferroxidase Activity and ^{59}Fe Transport in Intestinal Mucosal Cytosol. The enzyme recently isolated and purified from intestinal mucosal homogenates, which promotes the oxidation and incorporation of iron into transferrin, has been identified as intestinal xanthine oxidase (Topham et al., 1981). The dietary administration of tungsten to rats has previously been shown to result in marked inactivation of the xanthine oxidase activity in the liver, kidney, and intestine (Johnson et al., 1974a). This inactivation of xanthine oxidase has been demonstrated to specifically result from the inhibition of the incorporation of molybdenum into newly synthesized enzyme (Johnson et al., 1974b). In addition, these studies demonstrated that the effect of tungsten in rats is extremely specific and that tungsten-treated rats lack any signs or symptoms of toxicity from the metal itself. Thus, tungsten-treated rats provided a convenient mechanism for assessing whether xanthine oxidase, the intestinal source of ferroxidase activity, could be involved in the intestinal mucosal processing of dietary ionic iron. Control, low-iron, and tungsten-treated rats were intragastrically administered $^{59}FeSO_4$. Animals were sacrificed 10 and 30 min after dosing, and the ^{59}Fe in the blood serum, the ^{59}Fe in the mucosal cytosol, and the ferroxidase activity of the mucosal cytosol were analyzed and compared (Table I). Tungsten-treated rats had greatly reduced specific ferroxidase activity, increased retention of ^{59}Fe in the mucosal cytosol, and decreased transport of ^{59}Fe to blood as compared to control animals. In contrast, low-iron animals had greater specific ferroxidase activity, less retention of ^{59}Fe in the mucosal cytosol, and increased transport of ^{59}Fe to blood as compared to control animals.

Table I: Relationship of Mucosal Ferroxidase Activity and Radioactive Iron in Mucosal Cytosol and Blood Serum

animal group	time of sacrifice after administering ^{59}Fe[a] (min)	specific mucosal ferroxidase activity [μM Fe^{3+}-Tf[b] min^{-1} (mg of protein)$^{-1}$]	radioactivity in mucosal cytosol (cpm/0.5 mL)	radioactivity in blood serum (cpm/0.5 mL)
control	10	13.6	8 600	27 100
	30	13.2	13 600	75 600
low iron	10	16.4	7 200	91 700
	30	24.8	4 800	141 700
tungsten treated	10	1.27	12 000	16 900
	30	1.21	19 700	52 800

[a] Animals received 4 μCi of ^{59}Fe/150 g body weight as $FeSO_4$ in 0.005 M HCl in normal saline. [b] Tf = transferrin.

Table II: Distribution of ^{59}Fe in Mucosal Cytosol

animal group[a]	total ^{59}Fe in mucosal cytosol applied to column[b] (cpm)	^{59}Fe recovered in mucosal ferritin (cpm)	^{59}Fe recovered in mucosal transferrin (cpm)	% total ^{59}Fe recovered in both proteins
control	347 880	144 250	118 510	75.6
low iron	258 360	79 222	125 810	79.3
tungsten treated	478 640	300 830	61 400	76.0

[a] Animals sacrificed 10 min after intragastric administration of ^{59}Fe. [b] Columns of Sephadex G-200 (2.6 × 60 cm) used for gel filtration of 10-mL samples of mucosal cytosol.

Analysis of the ^{59}Fe Labeling of Mucosal Ferritin and Mucosal Transferrin. Additional animals from each diet group were administered ^{59}Fe intragastrically and sacrificed after 10 min, and mucosal cytosol was prepared. The separation of mucosal ferritin and mucosal transferrin from these mucosal cytosol prreparations was performed on a column of Sephadex G-200 which resulted in complete resolution of these two ^{59}Fe-labeled proteins and, thus, permitted calculation of the total ^{59}Fe radioactivity associated with each protein (Table II).

As observed in the previous experiment (Table I), the mucosal cytosol from tungsten-treated rats contained more ^{59}Fe than that from control animals. Tungsten-treated animals had double the amount of ^{59}Fe bound to mucosal ferritin and half the amount of ^{59}Fe bound to mucosal transferrin as compared to control animals. In contrast, low-iron animals had half the amount of ^{59}Fe in mucosal ferritin but an equivalent amount of ^{59}Fe in mucosal transferrin as compared to the control animals. In all animal groups, 75–80% of the total ^{59}Fe in the mucosal cytosol sample applied to the Sephadex G-200 column was recovered in the mucosal ferritin and mucosal transferrin eluted from the column. With respect to the remaining 20–25%, approximately 5–10% of the total ^{59}Fe applied to the column eluted as a peak prior to mucosal ferritin and 10–15% eluted in a peak considerably later than mucosal transferrin. The distribution of ^{59}Fe between these two minor components did not differ in control, low-iron, and tungsten-treated animals. The larger of these minor ^{59}Fe-labeled components has previously been tentatively identified as membrane fragments not removed during the centrifugation whereas the smaller of the minor ^{59}Fe-labeled components has previously been tentatively identified as a nonprotein, low molecular weight, chelate of iron (Halliday et al., 1976).

Similar gel-filtration studies of mucosal cytosol from animals sacrificed 30 min rather than 10 min after intragastric administration of ^{59}Fe indicated that the even greater retention of ^{59}Fe in the mucosal cytosol of these tungsten-treated animals was the result of additional accumulation of ^{59}Fe in the mucosal ferritin compartment with little if any change in the ^{59}Fe associated with mucosal transferrin.

Polyacrylamide Gel Electrophoresis of the Mucosal Cytosols. Samples of the mucosal cytosols obtained from each animal group were subjected to polyacrylamide gel electrophoresis to determine whether differences in the relative amounts of mucosal ferritin and mucosal transferrin could account for the observed changes in the distribution of ^{59}Fe between these two proteins. Differences in the mucosal cytosol content of the two proteins were observed for the three animal groups; however, the differences were not altered within a given group by the time of sacrifice after ^{59}Fe administration. The mucosal cytosols of both the tungsten-treated and low-iron

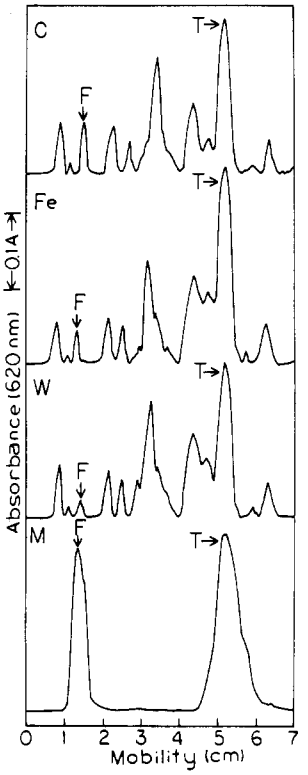

FIGURE 1: Polyacrylamide gel electrophoreses of mucosal cytosol samples. The polyacrylamide gel electrophoreses of the mucosal cytosol samples were performed just as described under Experimental Procedures. C, Fe, and W designate electrophoretic profiles of proteins in the mucosal cytosol of control, low-iron, and tungsten-treated rats, respectively. M, electrophoretic markers; F, ferritin; T, transferrin.

Table III: Time Course of the Effect of Dietary Tungsten

time on tungsten-supplemented diet[a] (days)	specific mucosal ferroxidase activity [μM Fe^{3+}-Tf min^{-1} (mg of protein)$^{-1}$]	cpm ferritin/ (cpm transferrin)
0	15.0	1.28
1.5	4.72	1.94
3.0	1.91	4.52
refed control diet, 2 days	8.92	1.44

[a] Animals received 4 μCi of ^{59}Fe/150 g body weight as $FeSO_4$ in 0.005 M HCl in normal saline; all animals were sacrificed 30 min after receiving ^{59}Fe.

animals contained much less mucosal ferritin than did the mucosal cytosol of control animals (Figure 1). The mucosal transferrin contents of the mucosal cytosols of control and tungsten-treated animals were identical; however, the mucosal transferrin of the low-iron animals was slightly greater. Since the actual amount of mucosal ferritin was much smaller in tungsten-treated animals than in control animals and the amount of mucosal transferrin was equivalent in the two groups, the increased ^{59}Fe labeling of mucosal ferritin and decreased ^{59}Fe labeling of mucosal transferrin in tungsten-treated animals cannot simply result from changes in the relative size of these two protein compartments.

Time Course of the Effect of Tungsten Treatment. Previous studies (Johnson et al., 1974a,b) have demonstrated that administration of tungsten to rats very rapidly decreases the xanthine oxidase activity of the liver, kidney, and small intestine. The $T_{1/2}$ for loss of xanthine oxidase activity in small intestine was 1.3 days. The effect of tungsten was rapidly reversible upon removal of tungsten from the diet or upon administration of additional molybdenum to the tungsten-treated animals. Animals which had been on the tungsten-supplemented diet for various time intervals were administered ^{59}Fe and sacrificed 30 min thereafter to determine if changes in the distribution of ^{59}Fe between mucosal ferritin and mucosal transferrin in the mucosal cytosol occurred in the same time frame as the rapid decrease in the ferroxidase activity of xanthine oxidase. The mucosal cytosol prepared from these animals was assayed for ferroxidase activity, and a sample of this mucosal cytosol was subjected to gel filtration on Sephadex G-200 to permit separation of mucosal ferritin and mucosal transferrin (Table III). After only 1.5 days on the tungsten diet, the specific ferroxidase activity of the mucosal cytosol had dropped to approximately 30% of the control, and the ratio of ^{59}Fe in mucosal ferritin to ^{59}Fe in mucosal transferrin had increased significantly. After 3 days on the tungsten-supplemented diet, the specific ferroxidase activity of the mucosal cytosol had decreased to approximately 10% of the control, and the ratio of ^{59}Fe in mucosal ferritin to ^{59}Fe in mucosal transferrin had increased by an even greater extent. When animals which had been on the tungsten-supplemented diet for 3 days were refed the control diet for 2 days, the specific ferroxidase activity of the mucosal cytosol returned to 60% of the control value, and the ratio of ^{59}Fe in mucosal ferritin to ^{59}Fe in mucosal transferrin decreased to a value slightly larger than that for the controls.

In Vitro Competition Studies with Apoferritin and Apotransferrin. The ability of apoferritin to compete with apotransferrin for Fe^{3+} formed from Fe^{2+} as a result of the ferroxidase activity of intestinal xanthine oxidase was investigated. The initial rate and total amount of iron incorporation into apotransferrin were analyzed in assay mixtures containing a

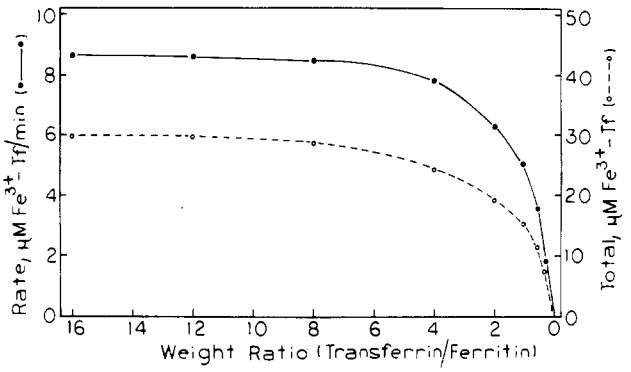

FIGURE 2: Effect of various concentrations of apoferritin on the incorporation of iron into apotransferrin catalyzed by intestinal xanthine oxidase. Assays of the ferroxidase activity of xanthine oxidase were conducted as described under Experimental Procedures except that assays contained amounts of apoferritin varying from 0.125 to 15 mg in addition to the 2 mg of apotransferrin included in each assay.

constant amount of apotransferrin and varying amounts of apoferritin. Neither the rate nor the total amount of iron incorporation into apotransferrin was significantly decreased until the weight ratio of apotransferrin to apoferritin in the assay mixture was decreased to 2 (Figure 2). Savin & Cook (1980), utilizing a highly sensitive two-site immunoradiometric assay, have demonstrated that, even in iron-loaded animals, the weight ratio of mucosal transferrin to mucosal ferritin is 4.5 and that this ratio increases to 5.9 in control animals and to 23.4 in iron-deficient animals. The electrophoretic studies described in the present paper also show that the ratio of mucosal transferrin to mucosal ferritin in control animals is considerably greater than 2.0 and is even larger in low-iron and tungsten-treated animals. This would suggest that virtually all the Fe^{3+} formed as a result of the ferroxidase activity of intestinal xanthine oxidase would be sequestered by mucosal transferrin unless the mucosal transferrin compartment in the mucosal cell approaches saturation.

Discussion

Unlike most other trace elements and nutrients, the capacity of the body to excrete iron is very limited, and in consequence, the iron content of the body is regulated primarily by absorption and not by excretion (Turnbull, 1974; Linder & Munro, 1977). Numerous studies [e.g., see Bothwell et al. (1968), Bannerman et al. (1962), and Charlton et al. (1965)] attest to the relationship between iron stores and iron absorption. Absorption is increased during periods of iron deficiency and decreased when body iron stores are in excess, and there is considerable evidence that the regulation of iron absorption occurs in pathways that operate within the duodenal mucosal cell (Granick, 1946; Crosby, 1963; Forth & Rummel, 1973). However, despite intensive investigation, the molecular mechanism by which changes in body iron stores are translated into alterations in the mucosal processing of iron is still not well understood.

Two major iron-binding proteins have been identified in the homogenized intestinal mucosa in several species. One of these proteins has been definitively identified as an isoferritin (Granick, 1946; Crosby, 1963; Manis & Schachter, 1964; Charlton et al., 1965; Pearson & Reich, 1969; Huebers et al., 1971, 1976; Worwood & Jacobs, 1971; Pollack et al., 1972; Sheenan & Frenkel, 1972; Halliday & Powell, 1973; Halliday et al., 1976; Valberg et al., 1977; El-Shobaki & Rummel, 1977; Savin & Cook, 1980). The second protein has properties very similar to, though not identical with, plasma transferrin, and this protein has been designated mucosal transferrin (Huebers

et al., 1971, 1976; Worwood & Jacobs, 1971; Halliday et al., 1976; Valberg et al., 1977; El-Shobaki & Rummel, 1977; Savin & Cook, 1980). Both of these proteins preferentially bind iron in the ferric oxidation state. Considerable evidence indicates that these two iron-binding proteins represent functionally distinct iron-binding compartments in the duodenal mucosa. Mucosa transferrin is postulated to mediate the transcellular transport of iron through the mucosal cell (Huebers et al., 1971; Pollack et al., 1972; Halliday et al., 1976; El-Shobaki & Rummel, 1977; Savin & Cook, 1980). This concept would be in keeping with the role of transferrin in plasma. Mucosal ferritin is postulated to be a storage compartment which binds iron that is rapidly transported to circulation by mucosal transferrin and constitutes a reservoir of storage iron within the mucosal cell (Charlton et al., 1965; Sheenan & Frenkel, 1972; Halliday & Powell, 1973; Halliday et al., 1976; Linder & Munro, 1977; El-Shobaki & Rummel, 1977; Savin & Cook, 1980). This role for mucosal ferritin would be consistent with the role that ferritin plays in other tissues such as liver and spleen.

Recent studies, monitoring the labeling pattern of these two mucosal proteins with radioiron and the actual changes in the mucosal contents of ferritin and transferrin in iron deficiency and iron overload, suggest that absorption is regulated by both of these mucosal iron binding proteins (El-Shobaki & Rummel, 1977; Savin & Cook, 1980). It has been postulated on the basis of these studies that the two proteins monitor different ends of the spectrum of iron status, mucosal transferrin being responsible for the increase of iron absorption in iron deficiency and mucosal ferritin being responsible for the inhibition of iron absorption when iron stores are large.

A non ceruloplasmin enzyme which promotes the oxidation and incorporation of iron into transferrin has recently been isolated and purified from intestinal mucosal homogenates and identified as intestinal xanthine oxidase (Topham, 1978; Topham el al., 1981). It has been postulated that intestinal xanthine oxidase, by promoting the oxidation and incorporation of iron into mucosal transferrin, could perform a function in iron absorption similar to the role that ceruloplasmin serves in the mobilization of iron from tissue stores. Several previous studies have demonstrated that the administration of substrates of xanthine oxidase to a variety of animals enhances intestinal iron absorption and increases the plasma iron concentration (Mazur et al., 1958; Cheney & Finch, 1960; Faelli & Esposito, 1970).

Tungsten-treated rats provided a convenient mechanism for assessing whether intestinal xanthine oxidase might be involved in the mucosal processing of dietary iron. The dietary administration of tungsten to rats has previously been shown to result in marked inactivation of the xanthine oxidase in the intestine (Johnson et al., 1974a). This inactivation of xanthine oxidase has been demonstrated to result specifically from the inhibition of the incorporation of molybdenum into newly synthesized enzyme (Johnson et al., 1974b). Furthermore, these studies demonstrated that the effect of tungsten in rats is extremely specific and that tungsten-treated rats lack any signs or symptoms of toxicity from the metal itself.

Tungsten-treated rats had greatly reduced mucosal ferroxidase activity, increased retention of ^{59}Fe in the mucosal cytosol, and decreased transport of ^{59}Fe to blood as compared to control and low-iron animals. These observations would be consistent with the proposal that intestinal xanthine oxidase, the mucosal source of ferroxidase activity, could play a role in the mucosal processing of dietary ionic iron. Comparison of the distribution of ^{59}Fe in the mucosal cytosol of the three animal groups revealed that the tungsten-treated rats, which possessed greatly decreased mucosal ferroxidase activity, had greatly decreased incorporation of ^{59}Fe into mucosal transferrin and greatly increased incorporation of ^{59}Fe into mucosal ferritin. This finding would be consistent with the proposal that intestinal xanthine oxidase facilitates the oxidation and incorporation of iron into mucosal transferrin. The electrophoretic studies demonstrated that even though the ^{59}Fe labeling of mucosal ferritin increased and the ^{59}Fe labeling of mucosal transferrin decreased, the actual amount of mucosal ferritin in the cytosol of tungsten-treated rats was less than that in control rats and the amount of mucosal transferrin the same as that in control rats. Thus, the altered distribution of ^{59}Fe in the mucosal cytosol of tungsten-treated rats could not result simply from alterations in the relative size of the two protein compartments.

The time course study of the effect of tungsten provided further support for the proposal that the altered distribution of ^{59}Fe in the mucosal cytosol of tungsten-treated rats was the result of the inactivation of xanthine oxidase. In these studies, the inactivation of xanthine oxidase upon the administration of dietary tungsten occurred very rapidly as previously reported (Johnson et al., 1974a). Large changes in the distribution of ^{59}Fe between mucosal ferritin and mucosal transferrin occurred in this same time interval. Furthermore, when the tungsten-treated rats were returned to the control diet for only 2 days, both the ferroxidase activity of xanthine oxidase and the distribution of ^{59}Fe between mucosal ferritin and mucosal transferrin returned to values which were close to those of control animals. The time course study also indicated that the initial reduction of the ferroxidase activity of xanthine oxidase to 30% of the control value in 1.5 days had a smaller effect on the ratio of ^{59}Fe in mucosal ferritin to ^{59}Fe in mucosal transferrin than the additional reduction of the activity from 30% to 10% of the control which occurred between 1.5 and 3 days. This may suggest that more activity is initially present in the mucosal cytosol than is required for normal rates of iron incorporation into mucosal transferrin. Thus, the 10% residual activity observed in the mucosal cytosol of the tungsten-treated rats could account for the finding that even though the incorporation of ^{59}Fe into mucosal transferrin in the tungsten-treated rats was markedly reduced it was not totally prevented. This is particularly interesting because a similar situation exists in terms of the incorporation of iron into plasma transferrin by ceruloplasmin. Less than 10% of the ferroxidase activity of ceruloplasmin normally present in blood plasma is required to ensure proper incorporation of iron into plasma transferrin and normal rates of iron turnover from tissue stores (Osaki & Johnson, 1969; Roeser et al., 1970; Osaki et al., 1971). The ability to facilitate iron mobilization from tissue stores is only one of many functions that plasma ceruloplasmin performs for the vertebrate organism (Frieden, 1980); it is possible that a similar situation exists in regard to the participation of xanthine oxidase in iron absorption.

It has been demonstrated that iron in the mucosa reacts as two chemically distinct forms, Fe^{2+} and Fe^{3+}, and that the Fe^{2+} pool is the precursor of the Fe^{3+} pool (Manis & Schachter, 1964). Ferritin possesses innate ferroxidase activity (Niederer, 1970; Macara et al., 1972; Crichton & Bryce, 1972) and, thus, has a mechanism for the acquisition of trivalent iron from the mucosal Fe^{2+} pool in the absence of intestinal xanthine oxidase. Hence, the greatly increased ^{59}Fe incorporation into mucosal ferritin in the cytosol of tungsten-treated rats could simply result from the ineffective acquisition of iron from the Fe^{2+} pool by mucosal transferrin in the absence of intestinal xan-

thine oxidase. However, previous studies (Mazur & Carleton, 1965) have provided evidence that xanthine oxidase acting as a dehydrogenase may be involved in the release of iron from ferritin stores. Although a large fraction of the iron sequestered by ferritin in the intestinal mucosa remains in the mucosal cell until it sloughs off the tip of the villus, there is evidence which suggests that some of the iron bound to mucosal ferritin may feed gradually into the blood stream (Charlton et al., 1965). Krenitsky & Tuttle (1978) recently found evidence for the presence of xanthine dehydrogenase and a catalytically and immunologically distinct xanthine oxidase in mouse small intestine. Both the oxidase and dehydrogenase require bound molybdenum for activity, and thus, both would be inactivated in the intestinal mucosa of tungsten-treated rats. Hence, the large increase in the ^{59}Fe bound to mucosal ferritin in the tungsten-treated rats as compared to control and low-iron animals could be the result of both ineffective acquisition of iron from the mucosal Fe^{2+} pool by mucosal transferrin in the absence of intestinal xanthine oxidase and ineffective release of iron from mucosal ferritin by intestinal xanthine dehydrogenase.

In summary, the results presented in this paper would support the proposal that intestinal xanthine oxidase, by promoting the incorporation of iron into mucosal transferrin, may perform a similar function in iron absorption as ceruloplasmin performs in iron mobilization. If so, this could explain why little interference is observed in the uptake of iron by intestinal mucosa, the processing of iron within the mucosal cell, and the regulation of intestinal iron absorption during copper and ceruloplasmin deficiency. It may also explain why administration of substrates of xanthine oxidase to a variety of animals enhances intestinal iron absorption.

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References

Bannerman, R. M., O'Brien, J. R. P., & Witts, L. J. (1962) *Blood 20*, 532–546.

Bothwell, T. H., Pirzo-Biroli, G., & Finch, C. H. (1968) *J. Lab. Clin. Med. 51*, 24–36.

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

Brittin, G. M., & Chee, Q. T. (1969) *J. Lab. Clin. Med. 74*, 53–59.

Charlton, R. W., Jacobs, P., Torance, J. O., & Bothwell, T. H. (1965) *J. Clin. Invest. 44*, 543–554.

Cheney, B., & Finch, C. A. (1960) *Proc. Soc. Exp. Biol. Med. 74*, 53–59.

Crichton, R. R., & Bryce, C. F. A. (1972) *Biochem. J. 130*, 36p.

Crosby, W. H. (1963) *Blood 22*, 441–449.

El-Shobaki, F. A., & Rummel, W. (1977) *Res. Exp. Med. 171*, 243–253.

Evans, J. L., & Abraham, P. A. (1973) *J. Nutr. 103*, 196–201.

Faelli, A., & Esposito, G. (1970) *Biochem. Pharmacol. 19*, 2551–2554.

Forth, W., & Rummel, W. (1973) *Physiol. Rev. 53*, 724–792.

Frieden, E. (1980) *Ciba Found. Symp. 79*, 93–124.

Granick, S. (1946) *J. Biol. Chem. 164*, 737–744.

Halliday, J. W., & Powell, L. W. (1973) *Clin. Chim. Acta 43*, 267–276.

Halliday, J. W., Powell, L. W., & Mack, U. (1976) *Br. J. Haematol. 34*, 237–250.

Huebers, H. (1975) in *Proteins of Iron Storage and Transport in Biochemistry and Medicine* (Crichton, R. R., Ed.) pp 381–388, North-Holland Publishing Co., Amsterdam.

Huebers, H., Huebers, E., Forth, W., & Rummel, W. (1971) *Life Sci. 10*, 1141.

Huebers, H., Huebers, E., Rummel, W., & Crichton, R. R. (1976) *Eur. J. Biochem. 66*, 447–455.

Johnson, D. A., Osaki, S., & Frieden, E. (1967) *Clin. Chem. (Winston-Salem, N.C.) 16*, 65.

Johnson, D. A., Osaki, S., & Frieden, E. (1970) *Clin. Chem. (Winston-Salem, N.C.) 13*, 142–149.

Johnson, J. L., Rajagopalan, K. V., & Cohen, H. J. (1974a) *J. Biol. Chem. 248*, 859–866.

Johnson, J. L., Waud, W. R., Cohen, H. J., & Rajagopalan, K. V. (1974b) *J. Biol. Chem. 249*, 5051–5061.

Krenitsky, T. A., & Tuttle, J. V. (1978) *Arch. Biochem. Biophys. 185*, 370–375.

Lee, G. R., Nacht, S., Lukens, J. N., & Cartwright, G. E. (1968) *J. Clin. Invest. 47*, 2058–2069.

Linder, M. C., & Munro, H. N. (1977) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36*, 2017–2023.

Macara, I. G., Hoy, T. G., & Harrison, P. M. (1972) *Biochem. J. 126*, 151–162.

Manis, J., & Schachter, D. (1964) *Am. J. Physiol. 207*, 893–900.

Mazur, A., & Carleton, A. (1965) *Blood 26*, 317–322.

Mazur, A., Green, S., Caha, A., & Carleton, A. (1958) *J. Clin. Invest. 37*, 1809–1817.

Niederer, W. (1970) *Experientia 26*, 218–220.

Osaki, S. (1966) *J. Biol. Chem. 241*, 5053–5059.

Osaki, S., & Johnson, D. A. (1969) *J. Biol. Chem. 244*, 5757–5758.

Osaki, S., Johnson, D. A., & Frieden, E. (1966) *J. Biol. Chem. 241*, 2746–2751.

Osaki, S., Johnson, D. A., Topham, R. W., & Frieden, E. (1970) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 29*, 695.

Osaki, S., Johnson, D. A., & Frieden, E. (1971) *J. Biol. Chem. 246*, 3018–3023.

Pearson, W. N., & Reich, M. B. (1969) *J. Nutr. 99*, 137–140.

Pollack, S., & Laskey, F. D. (1975) in *Proteins of Iron Storage and Transport in Biochemistry and Medicine* (Crichton, R. R., Ed.) pp 389–393, North-Holland Publishing Co., Amsterdam.

Pollack, S., Campana, T., & Arcaria, A. (1972) *J. Lab. Clin. Med. 80*, 322–332.

Ragan, H. A., Nacht, S., Lee, G. R., Bishop, C. R., & Cartwright, G. E. (1969) *Am. J. Physiol. 217*, 1320–1323.

Roeser, H. P., Lee, G. R., Nacht, S., & Cartwright, G. E. (1970) *J. Clin. Invest. 49*, 2408–2417.

Savin, M. A., & Cook, J. D. (1980) *Blood 56*, 1029–1035.

Sheenan, R. G., & Frenkel, E. P. (1972) *J. Clin. Invest. 51*, 224–231.

Topham, R. W. (1978) *Biochem. Biophys. Res. Commun. 85*, 1339–1345.

Topham, R. W., & Frieden, E. (1970) *J. Biol. Chem. 245*, 6689–6705.

Topham, R. W., & Johnson, D. A. (1974) *Arch. Biochem. Biophys. 160*, 647–654.

Topham, R. W., Woodruff, J. H., Neatrour, G. P., Calisch, M. P., Russo, R. B., & Jackson, M. R. (1980) *Biochem. Biophys. Res. Commun. 96*, 1532–1539.

Topham, R. W., Woodruff, J. H., & Walker, M. C. (1981) *Biochemistry 20*, 319–324.
Turnbull, A. (1974) in *Iron in Biochemistry and Medicine* (Jacobs, A., & Worwood, W., Eds.) pp 369–403, Academic Press, New York.
Valberg, L. S., Sorbie, J., & Ludwig, J. (1977) *Br. J. Haematol. 35*, 321–330.
Williams, D. M., Lee, G. R., & Cartwright, G. E. (1974) *Am. J. Physiol. 227*, 1094–1097.
Worwood, M., & Jacobs, A. (1971) *Life Sci. 10*, 1363–1373.

Reversible Denaturation of *Aequorea* Green-Fluorescent Protein: Physical Separation and Characterization of the Renatured Protein†

William W. Ward* and Stephen H. Bokman

ABSTRACT: The green-fluorescent protein (GFP) that functions as a bioluminescence energy transfer acceptor in the jellyfish *Aequorea* has been renatured with up to 90% yield following acid, base, or guanidine denaturation. Renaturation, following pH neutralization or simple dilution of guanidine, proceeds with a half-recovery time of less than 5 min as measured by the return of visible fluorescence. Residual unrenatured protein has been quantitatively removed by chromatography on Sephadex G-75. The chromatographed, renatured GFP has corrected fluorescence excitation and emission spectra identical with those of the native protein at pH 7.0 (excitation λ_{max} = 398 nm; emission λ_{max} = 508 nm) and also at pH 12.2 (excitation λ_{max} = 476 nm; emission λ_{max} = 505 nm). With its peak position red-shifted 78 nm at pH 12.2, the *Aequorea* GFP excitation spectrum more closely resembles the excitation spectra of *Renilla* (sea pansy) and *Phialidium* (hydromedusan) GFPs at neutral pH. Visible absorption spectra of the native and renatured *Aequorea* green-fluorescent proteins at pH 7.0 are also identical, suggesting that the chromophore binding site has returned to its native state. Small differences in far-UV absorption and circular dichroism spectra, however, indicate that the renatured protein has not fully regained its native secondary structure.

The green-fluorescent proteins (GFP)[1] are a small class of unusual chromoproteins found only among certain bioluminescent coelenterates (Morise et al., 1974; Morin, 1974; Cormier et al., 1974; Prendergast & Mann, 1978; Ward & Cormier, 1979; Ward, 1979, 1981). These accessory proteins function as ultimate bioluminescence emitters, accepting energy from enzyme-bound, excited-state oxyluciferin (Ward & Cormier, 1976, 1978). Depending upon the species, the mechanism of energy transfer involves either radiationless transfer or trivial transfer (Ward, 1979). Two coelenterate species have been intensively studied as representatives of these two types of bioluminescence energy transfer systems. In the sea pansy, *Renilla reniformis* (and related anthozoans), energy transfer is clearly radiationless and a biochemical mechanism involving transient protein–protein interaction between luciferase and the *Renilla* GFP has been demonstrated in vitro (Ward & Cormier, 1976, 1978; Hart et al., 1979). In the jellyfish, *Aequorea aequorea*,[2] in vivo energy transfer appears to be a radiative process (Johnson et al., 1962; Ward, 1979, 1981) mediated by close physical packing of *Aequorea* GFP with the calcium-activated photoprotein aequorin (Shimomura et al., 1962, 1963) into specialized photocytes that line the perimeter of the jellyfish umbrella (Davenport & Nicol, 1955).

Green-fluorescent proteins from *Aequorea* and *Renilla* (abbreviated A-GFP and R-GFP, respectively) have been physically characterized as acidic, globular proteins with similar amino acid compositions and monomer molecular weights of 27 000–30 000 (Morise et al., 1974; Ward, 1979). *Aequorea* GFP exists as the monomer in dilute solution while the native form of R-GFP is a dimer of identical subunits stabilized by strong, noncovalent interactions. Their fluorescence emission spectra (λ_{max} = 508–509 nm), quantum efficiencies (78–80%), and fluorescence polarization values (0.4–0.5) are each very similar (Wampler et al., 1971; Cormier et al., 1973; Morise et al., 1974; Ward & Cormier, 1979; Prendergast, 1980), suggesting that both proteins contain the same chromophore. However, A-GFP has absorption and excitation maxima near 395 nm (Morise et al., 1974), and R-GFP has a major peak at 498 nm (Wampler et al., 1971; Ward & Cormier, 1979). Despite the large (103-nm) difference between absorption maxima, the fully denatured proteins (6 M guanidine hydrochloride, 90 °C) have identical absorption peaks in acid (383–384 nm) and base (447–448 nm) and a single isosbestic point in the visible region at 405 nm (Ward et al., 1980). It has been concluded that the spectral differences noted above for the native proteins are the result of unique noncovalent interactions between a common chromophore and differing apoprotein environments (Ward et al., 1980).

From papain digests of A-GFP, Shimomura has purified a chromopeptide with the same spectral characteristics as the

† From the Department of Biochemistry and Microbiology, Cook College, New Jersey Agricultural Experiment Station, Rutgers University, New Brunswick, New Jersey 08903. *Received April 29, 1982.* New Jersey Agricultural Experiment Station Publication No. D-01102-1-82. This work was supported by State funds and by grants to W.W.W. from the Rutgers Research Council (07-2312 and 07-2271) and the Department of Health, Education, and Welfare (Biomedical Research Support Grant PHS RR 7058-13).

[1] Abbreviations: GFP, green-fluorescent protein; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Gdn·HCl, guanidine hydrochloride; CD, circular dichroism.

[2] The species of *Aequorea* common to the waters around Friday Harbor, WA, has been referred to both as *A. aequorea* and *A. forskalea*; see Johnson & Shimomura (1978) for a discussion of the nomenclature.

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