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Purification and Characterization of the Intestinal Promoter of Iron(3+)–Transferrin Formation†

Richard W. Topham,* James H. Woodruff, and Mark C. Walker

ABSTRACT: The nonceruloplasmin enzyme located in the intestinal mucosa which promotes the incorporation of iron into transferrin has been resolved into a small, heat-stable component and a heat-labile protein component. The small, heat-stable component was purified from the high-speed supernatant of intestinal mucosal homogenates by ion-exchange chromatography and gel filtration and identified as xanthine. The heat labile protein component was purified from the high-speed supernatant of intestinal mucosal homogenates by heat treatment, gel filtration, and ion-exchange chromatography. The physical, spectral, and kinetic properties of the heat-labile protein component strongly suggest that it is xanthine oxidase. By promotion of the oxidation and incorporation of iron into transferrin, intestinal xanthine oxidase could perform a similar function in iron absorption as ceruloplasmin serves in the mobilization of iron from liver stores.

Strong evidence from several laboratories (Osaki & Johnson, 1969; Ragan et al., 1969; Osaki et al., 1970, 1971; Roeser et al., 1970; Evans & Abraham, 1973; Williams et al., 1974) has established that ceruloplasmin facilitates the mobilization of iron from liver stores. Considerable evidence suggests that ceruloplasmin facilitates iron mobilization by promoting the oxidation and incorporation of iron into transferrin (Osaki et al., 1966, 1970, 1971; Osaki, 1966; Osaki & Johnson, 1969). Although a physiological role for ceruloplasmin has been demonostrated in iron mobilization, Brittin & Chee (1969) found no relationship between ceruloplasmin and the intestinal absorption of iron. The dietary iron that traverses the intestinal mucosal cell must be eventually incorporated into transferrin for transport in the blood. The identification of a nonceruloplasmin enzyme in the intestinal mucosa that promotes the incorporation of iron into transferrin has been described in a preliminary report (Topham, 1978), and it has been postulated that this intestinal enzyme could serve a similar function in iron absorption as ceruloplasmin serves in iron mobilization. The present paper describes the purification and characterization of this enzyme system from rabbit intestinal mucosa. Data are presented which strongly suggest that this intestinal enzymatic promoter of Fe^{3+}–transferrin formation is xanthine oxidase.

Experimental Procedures

Materials

Rabbit Intestinal Mucosa. Frozen intestinal mucosa from the duodenum of young rabbits was purchased from Pel-Freez Biologicals, Inc., Rogers, AR.

Apotransferrin. 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). This apotransferrin was 98–99% pure as determined by polyacrylamide gel electrophoresis.

Homogenizing Media. High purity, crystalline sucrose was dissolved in deionized, glass-distilled water to yield a concentration of 0.25 M. Sodium azide, 0.02% (w/v), was added to the sucrose solution to prevent microbial growth. A previous report (Topham, 1978) indicated that the intestinal enzyme was insensitive to azide. The pH of this solution was adjusted to 7.4 with dilute NH_4OH. This homogenizing medium was used to prepare the intestinal extracts used for preliminary studies of the intestinal enzyme system and for the isolation and identification of the small, heat-stable component of the intestinal enzyme system.

The homogenizing medium used to prepare intestinal extracts for the purification and characterization of the heat-labile protein component was 0.05 M Hepes[1] buffer, pH 7.5. Better recoveries of the heat-labile component were obtained during the purification procedure with the Hepes buffer system.

Column Chromatographic Materials. Bio-Gel P-2 (Bio-Rad Laboratories, Richmond, CA) was swollen and equilibrated in deionized, glass-distilled water. AG1-X2 anion-exchange resin (Bio-Rad Laboratories, Richmond, CA) was obtained in the acetate form and was washed extensively in deionized, glass-distilled water prior to use. AG 50W-X2 cation-exchange resin (Bio-Rad Laboratories, Richmond, CA) was obtained in the hydrogen form and was prepared as described by Bergmann & Dikstein (1958). DEAE-Sephadex A-50 and Sephadex G-200 (Pharmacia Fine Chemicals, Pis-

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[1] Abbreviations used: Hepes, *N*-2-(hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DEAE, diethylaminoethyl; Tf, transferrin; S-105, supernatant of intestinal mucosal homogenates following centrifugation at 105000*g* for 1 h.

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cataway, NJ) were swollen and equilibrated with 0.05 M Hepes buffer, pH 7.5.

Molecular Weight Standards, Thin-Layer Chromatographic Standards, Other Proteins, and Substrates. Protein standards (ferritin, catalase, aldolase, ovalbumin, chymotrypsinogen A, and ribonuclease A) for the molecular weight determination by gel filtration were purchased from Pharmacia Fine Chemicals, Piscataway, NJ. High purity milk xanthine oxidase, superoxide dismutase, catalase, cytochrome *c*, xanthine, hypoxanthine, allopurinol, and uric acid were purchased from Sigma Chemical Co., St. Louis, MO.

Methods

Preparation of Intestinal Mucosal Extracts. A 20% (w/v) homogenate of rabbit intestinal mucosa was prepared with either the 0.25 M sucrose or the Hepes homogenizing medium in a power-driven homogenizer, with the pestle rotating at 1000 rpm for 1 min. The resulting homogenates were centrifuged at 15000*g* for 20 min. The supernatants obtained were passed through glass wool to remove any floating lipid material and then centrifuged at 105000*g* for 1 h. The high-speed supernatants, termed S-105 in this report, were carefully decanted from the microsomal pellets. The S-105 could be lyophilized and the lyophilized material was stored at −20 °C for a period of 3 weeks without any significanct loss of activity.

Enzyme Assays. The enzymatic oxidation and incorporation of iron into transferrin was measured spectrophotometrically at 460 nm, where Fe^{3+}–transferrin exhibits maximal absorbance. This spectrophotometric assay for Fe^{3+}–transferrin formation has been described in detail and validated in numerous previous reports (Osaki et al., 1966, 1971; Osaki, 1966; Johnson et al., 1967; Topham & Frieden, 1970; Topham & Johnson, 1974; Topham, 1978). In these studies, unless otherwise specified, each assay cuvette (1.5 mL capacity) contained 0.350 mL of a 0.6 M acetate buffer, pH 6.0, 0.250 mL of a 2% (w/v) apotransferrin solution, 0.300 mL of a 4×10^{-4} M ferrous ammonium sulfate solution, and 0.100 mL of the appropriate enzyme preparation. In assays requiring the addition of the small, heat-stable component, or a substitute thereof, 0.1 mL of a 1 mM solution of the component or the substitute was added to the assay prior to the addition of the enzyme. A control containing no enzyme solution was included with each assay to determine the nonenzymatic rate of Fe^{3+}–transferrin formation. This value was always subtracted from the observed enzymatic rate to obtain the true enzymatic rate. In all assays, the value for the nonenzymatic rate of Fe^{3+}–transferrin formation was less than 5% of the enzymatic rate.

Spectrophotometric and Fluorometric Measurements. All enzymatic assays and other spectrophotometric measurements were performed on a Beckman Acta Model C-III spectrophotometer equipped with a 0.1 absorbance unit scale. Protein elution from columns was monitored at 280 nm with an ISCO Model UA-5 absorbance monitor equipped with a type 6 optical unit. Spectrofluorometric measurements were carried out with a J4-8960 Aminco Bowman spectrofluorometer.

Protein Concentrations. Protein concentrations were determined by the Bio-Rad protein assay which is based on the protein dye binding principle described by Bradford (1976).

Ultrafiltration. Protein solutions were concentrated by using Amicon ultrafiltration cells, models 202, 52, 12, or M-3, equipped with PM-30 or YM-10 Diaflo ultrafiltration membranes.

Thin-Layer and Paper Chromatography. Thin-layer chromatographic analyses were performed on Baker-flex cellulose thin-layer sheets (J. T. Baker Chemical Co., Phillipsburg, NJ) with $CH_3OH/HCl/H_2O$ (70:20:10 v/v) as developing solvent. Paper chromatographic analyses were performed on Whatman No. 1 filter paper with 16% NH_4HCO_3 (w/v), pH 8.1, as the developing solvent (Wilson & Wilson, 1962). The positions of components on paper and thin-layer chromatograms were detected by utilizing a mineralight equipped with both long-wavelength and short-wavelength ultraviolet emission (model UVSL-25, Ultraviolet Products, Inc., San Gabriel, CA).

Estimation of Molecular Weights. A column (1.6 × 84 cm) of Sephadex G-200 was prepared and equilibrated with 0.05 M Hepes buffer, pH 7.5. Samples (1 mL, 5.0 mg of protein/mL) of six standard proteins of known molecular weight (ferritin, catalase, aldolase, ovalbumin, chymotrypsinogen A, and ribonuclease A) were passed through this column and the elution volumes (V_e) determined. The void volume (V_0) was determined by the passage of blue dextran through the column. Linear regression analysis of the ratios of elution volume to void volume (V_e/V_0) vs. the logarithms of the molecular weights of the standard proteins had a correlation coefficient of 0.993. The molecular weights of the purified intestinal enzyme and purified xanthine oxidase were estimated by comparing their V_e/V_0 ratios with those of the standard proteins.

Polyacrylamide Gel Electrophoresis. Prepolymerized Biophore 7.5% polyacrylamide separating gels (Bio-Rad Laboratories, Richmond, CA) were equilibrated with 0.188 M Tris–glycine buffer, pH 8.9, prior to use. A 40-μL aliquot of Biotracking dye (Bio-Rad Laboratories, Richmond, CA) containing 30% sucrose was added to 160 μL of protein samples (0.2–4 mg of protein/mL) to obtain a final volume of 200 μL.

Samples of these preparations (20–100 μL) were applied to the tops of the gels. The samples were run into the separating gels at 80 V for 30 min. The electrophoresis was completed at 150 V for 3 H. The gels were then fixed with a solution of 2-propanol/H_2O/acetic acid (40:50:10 v/v), stained with coomassie blue, and destained with 7% acetic acid. The stained gels were scanned for protein at 280 nm with an ISCO Model 1310 gel-scanning attachment for the ISCO Model UA-5 absorbance monitor.

Steady-State Kinetic Analyses. The K_m values of Fe^{2+}, hypoxanthine, and xanthine for the intestinal promoter of iron incorporation into transferrin and for a sample of authentic xanthine oxidase were determined in the assay system utilized to measure enzymatic formation of Fe^{3+}–transferrin. For determination of the K_m for Fe^{2+}, the initial rate of Fe^{3+}–transferrin formation was measured at initial Fe^{2+} concentrations from 10 to 120 μM with a constant hypoxanthine concentration of 100 μM. For determination of the K_m values for hypoxanthine and xanthine, the initial rate of Fe^{3+}–transferrin formation was measured at initial hypoxanthine or xanthine concentrations from 2 to 50 μM with a constant Fe^{2+} concentration of 120 μM. The K_m values for Fe^{2+}, hypoxanthine, and xanthine for the purified intestinal enzyme and authentic xanthine oxidase were computed by linear regression analyses of the reciprocals of the initial velocities and the reciprocals of the Fe^{2+}, hypoxanthine, or xanthine concentrations. In all computations, correlation coefficients of 0.995–0.999 were obtained.

Inhibition by Allopurinol. Enzyme assay mixtures were prepared as previously described with either 0.1 mL of the purified intestinal enzyme (0.2 mg of protein/mL) or 0.1 mL of authentic xanthine oxidase (0.2 mg of protein/mL). To each assay cuvette was added 0.1 mL of a 0.1 mM solution

of allopurinol. The assay mixtures containing allopurinol were allowed to incubate 1 min prior to addition of 0.1 mL of a 1 mM solution of hypoxanthine, which initiated the enzymatic formation of Fe^{3+}–transferrin. The degree of inhibition by allopurinol was determined by comparing initial rates of Fe^{3+}–transferrin formation in assays containing allopurinol with identical assays containing 0.1 mL of distilled H_2O instead of the allopurinol solution.

Uric Acid Formation. Uric acid formation by xanthine oxidase is most conveniently determined spectrophotometrically by following the increase in absorbance at 290 nm upon the enzymatic oxidation of hypoxanthine (Kalckar, 1947). To determine if hypoxanthine was oxidized and uric acid formed by the purified intestinal enzyme, assay mixtures were prepared as previously described except that 0.25 mL of distilled water was substituted for the 0.25 mL of apotransferrin. Without this substitution, it was not possible to monitor uric acid formation at 290 nm because of the large absorbance of the apotransferrin at this wavelength. Each assay contained 100 μM hypoxanthine and 0.1 mL of purified intestinal enzyme (0.2 mg of protein/mL) or 0.1 mL of authentic xanthine oxidase (0.2 mg of protein/mL).

Superoxide Formation. Superoxide formed enzymatically by xanthine oxidase has been shown to reduce cytochrome *c* (McCord & Fridovich, 1968). The reduction of cytochrome *c* by superoxide is potently inhibited by superoxide dismutase (McCord & Fridovich, 1969). Cytochrome *c* reduction and the inhibition of cytochrome *c* reduction by superoxide dismutase were used to determine whether superoxide was produced during the reoxidation of the purified intestinal enzyme by molecular oxygen.

Specifically, assay mixtures containing 0.7 mL of 0.6 M acetate buffer, pH 6.0, 0.2 mL of cytochrome *c* (5×10^{-4} M), and 0.4 mL of the purified intestinal enzyme (0.77 mg/mL) were incubated 10 min at 30 °C to obtain a base line at 550 nm. After this 10-min preincubation, 200 μL of hypoxanthine (1 mM) was added, and the change in absorbance at 550 nm was followed. In a second set of assays, 200 μL of buffer was replaced by 200 μL of superoxide dismutase (12 mg/mL) and cytochrome *c* reduction analyzed as in the preceding set of assays.

Results

Detection of the Requirement for a Small, Heat-Stable Component. Previous studies (Topham, 1978) demonstrated that the intestinal promoter of Fe^{3+}–transferrin formation was enzymatic in nature and located in the supernatant following centrifugation of intestinal mucosa homogenates at 105 000*g* (S-105). Initial attempts to purify the enzyme from the S-105 fraction by gel filtration proved unsuccessful. Gel filtration of the S-105 fraction on columns of various G-type Sephadexes, P-type Bio-Gels, or agarose gels resulted in the complete loss of activity. Dialysis of the supernatant also resulted in a dramatic decrease in activity. These observatons suggested that a loosely bound, low molecular weight component, which was being removed during gel filtration and dialysis procedures, might be required for activity. For investigation of this possibility, a 5-mL sample of the S-105 fraction was concentrated to 2 mL by ultrafiltration and this initial filtrate saved. The concentrated S-105 was diluted to 5 mL and reconcentrated to 2 mL. This dilution and concentration procedure was repeated a second time. The materials retained by the PM-30 filter following the repetitive ultrafiltrations and the initial filtrate were individually tested for activity and protein content. A sample of the retained material was combined with an equal volume of the initial filtrate, and this recombined sample was tested for activity and protein. The activity of the repetitively ultrafiltered S-105 was markedly decreased. The initial filtrate alone had no activity. However, when the ultrafiltered S-105 was combined with the initial filtrate, the specific activity of this recombined sample was equivalent to the specific activity of the original S-105. These findings strongly suggested that a loosely bound, low molecular weight component was required for activity. A sample of the S-105 was applied to a column of Sephadex G-25. Three bands of materials possessing absorbance at 280 nm were eluted from the column (Figure 1). When individual fractions from each band were assayed for protein, only those fractions comprising the first band (I) contained protein. The fractions comprising each of the three bands were combined and concentrated by lyophilization. The solid material obtained from the lyophilization of each of these samples was reconstituted by dissolving in a minimal amount (~2 mL) of distilled water. The specific activities of the individual samples and various combinations of equal volumes of the samples were determined (Table I). None of the individual samples had activity; however, when samples of bands I and III were combined, a specific activity equivalent to the S-105 before gel filtration on Sephadex G-25 was obtained. Heat treating a sample of band I resulted in complete loss of this activity whereas heat treatment of a sample of band III had no effect. Experiments were then conducted in which the amount of one of the components in the assay mixture was held constant and the other varied (Figure 2). A linear relationship between the activity and amount of band I was

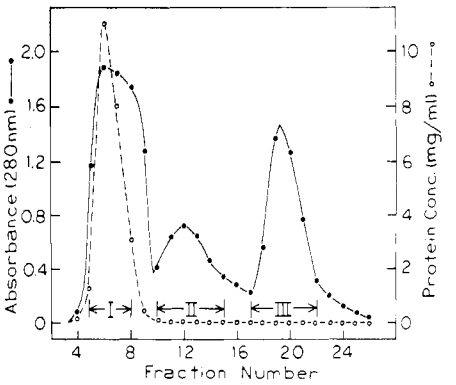

FIGURE 1: Separation of the protein and small molecular weight components by gel filtration. A 1-g sample of lyophilized S-105 from rabbit intestinal mucosal homogenates was redissolved in 3 mL of distilled water. The activity and protein content of the resulting solution were determined. A sample of this solution (2.7 mL) was applied to a column (1.6 × 30 cm) of Sephadex G-25. Fractions of 4.3 mL were collected. Each fraction exhibiting absorbance at 280 nm was assayed for protein content. The fractions comprising each of the three individual bands were pooled and concentrated by lyophilization. The specific activities of the individual bands and various combinations of the bands were determined (Table II).

Table I: Restoration of Activity following Gel Filtration of S-105

sample	sp act. [μM Fe^{3+}-Tf min^{-1} (mg of protein)$^{-1}$]
S-105 before gel filtration	1.68
band I	0
band II	0
band III	0
bands I + II	0
bands I + III	1.76
bands II + III	0
boiled band I[a] + band III	0
band I + boiled band III[a]	1.64

[a] Heated at 100 °C for 5 min.

Table II: Analysis of the Small, Heat-Stable Component

sample	UV spectra, λ_{max} (nm)			fluorescence (nm)		R_f		sp act. of intestinal enzyme[a]	substrate for xanthine oxidase[b]
	pH 2.0	pH 7.2	pH 10.0	λ_x	λ_e	TLC	paper		
active band from	263	271	282	270	335				
P-2 column	228	232		240	335	0.49	0.55	2.45	yes
authentic	261	270	283	270	335				
xanthine	230	234		240	335	0.48	0.55	2.52	yes

[a] Reported as μM Fe^{3+}–Tf min^{-1} (mg of protein)$^{-1}$. [b] Measured as uric acid formation.

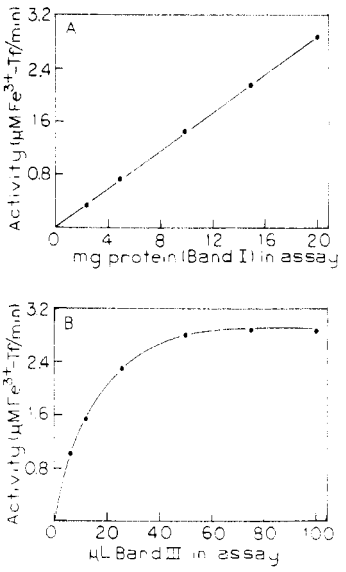

FIGURE 2: Relationship between the activity and the amounts of bands I and III obtained by gel filtration. (A) A 0.1-mL aliquot of concentrated band III, prepared as described in the legend to Figure 1, was added to each assay, and the amount of concentrated band I, prepared as described in the legend to Figure 1, was varied. (B) A 0.1-mL sample of concentrated band I was added to each assay, and the amount of concentrated band III was varied.

observed whereas a hyperbolic relationship between the activity and amount of band III was observed. These findings further substantiated that the intestinal promoter of Fe^{3+}–transferrin formation was composed of a heat-labile protein component and a small, heat-stable component that could be completely resolved by gel filtration.

Purification and Identification of the Small, Heat-Stable Component. To facilitate purification and identification, samples containing the small, heat-stable component were prepared on a larger scale. A 50-mL aliquot of the S-105 fraction was applied directly to a large Sephadex G-25 column (3 × 40 cm), and the column was developed with the sucrose homogenizing solution. The elution pattern of ultraviolet-absorbing components from this column was identical with that observed with the smaller scale Sephadex G-25 column (Figure 1). The fractions containing the small, heat-stable component (band III) were combined and lyophilized, and the residue was redissolved in 10 mL of distilled H_2O. This sample was chromatographed on a column (4.5 × 80 cm) of Bio-Rad AG 1-X2 to remove the sucrose. Sucrose eluted with distilled H_2O and the ultraviolet-absorbing material which contained the small, heat-stable component eluted with 0.3 M NaCl. The latter material was lyophilized, the residue redissolved in 5 mL of distilled H_2O, and the pH of the solution adjusted to 2.0. Furthermore purification was achieved by chromatography of this solution on a column (1.2 × 12 cm) of Bio-Rad AG 50W-X2 cation-exchange resin. The column was sequentially treated with 0.1 N HCl, H_2O, and 10% NH_4OH. Two bands of ultraviolet-absorbing material were eluted from this column, one with 0.1 N HCl and the other with 10% NH_4OH. In order to determine which contained the small, heat-stable component, each of these column fractions was lyophilized and redissolved in 0.05 M acetate buffer, pH 6.0. Only the material eluted with 10% NH_4OH was capable of restoring enzymatic activity. The purification of the small, heat-stable component was completed by gel filtration of this sample on a column (1.3 × 120 cm) of Bio-Gel P-2. Four bands of ultraviolet-absorbing material were eluted from this column with distilled H_2O. After lyophilization and reconstitution, only the second of these bands resulted in the restoration of enzymatic activity. Thin-layer and paper chromatographic analyses of this sample indicated that it contained a single component.

Spectrally and chromatographically, the purified small, heat-stable component appeared virtually identical with xanthine (Table II). To conclusively establish the identity of the small, heat-stable component, a solution of authentic xanthine was prepared with a concentration (based on the ultraviolet absorption at 270 nm) equivalent to that of the solution of the material purified form the intestinal homogenate. The ability of the authentic xanthine to restore enzymatic activity was equivalent to that of the purified, small, heat-stable component. Furthermore, the purified, small, heat-stable component was a substrate for xanthine oxidase.

Purification of the Heat-Labile Component. Hypoxanthine, as well as xanthine, was capable of restoring enzyme activity to the heat-labile protein component following gel filtration on Sephadex G-25. Since hypoxanthine is considerably more water soluble than xanthine, a 1 mM solution of hypoxanthine was prepared, and 0.1 mL of this solution was added to enzyme assays to restore activity to the heat-labile protein component during the course of its purification. One fourth of this amount was sufficient to yield a maximal initial rate of iron incorporation into transferrin when added to enzyme assays containing the heat-labile protein component.

Routinely, 100 mL of the S-105, prepared with the Hepes homogenizing medium, served as the starting material for the purification of the heat-labile protein component. Heat treatment of the S-105 at 60 °C for 20 min, followed by centrifugation to remove denatured protein, resulted in considerable initial purification with little loss of activity. Treatment at higher temperatures or for longer periods resulted in appreciable losses of activity. The heat-treated S-105 was concentrated to 50 mL by ultrafiltration and applied to a column (5 × 55 cm) of Sephadex G-200 which was developed with 0.05 M Hepes buffer, pH 7.5. The enzymatically active fractions from this column were combined, and the combined sample was applied to a column (2.6 × 12 cm) of DEAE-Sephadex which was developed with a stepwise gradient of NaCl (0, 0.1, 0.15, 0.3, and 0.6 M) in 0.05 M Hepes, pH 7.5. The enzyme activity eluted with 0.15 M NaCl, and the bulk of the inactive contaminating protein eluted with 0.3 and 0.6 M NaCl. The enzymatically active material from the DEAE-Sephadex column was concentrated to 5 mL by ultrafiltration and subjected to two cycles through an additional

Table III: Purification of the Heat-Labile Protein Component

purification step	total protein (mg)	total activity (μM Fe^{3+}–Tf min^{-1})	sp act. [μM Fe^{3+}–Tf min^{-1} (mg of protein)$^{-1}$]	x-fold purification	% recovery
S-105	1621	5000	3.08	1	100
heat-treated S-105	339	4880	14.4	4.66	97.5
G-200$_I$	29.3	3810	130	42.2	76.3
DEAE-Sephadex	3.14	2372	755	245	47.5
G-200$_{II}$	0.772	1212	1578	508	31.4
G-200$_{III}$	0.283	1040	3668	1189	20.8

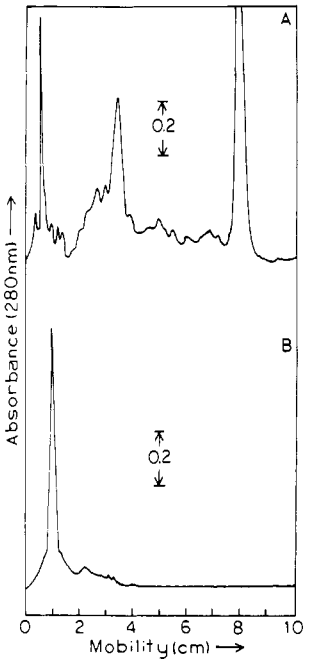

FIGURE 3: Polyacrylamide gel electrophoresis of heat-treated S-105 and the purified, heat-labile, protein component. The polyacrylamide gel electrophoreses were performed just as described under Methods. (A) S-105 following heat treatment. (B) Purified, heat-labile, protein component after complete purification procedure (Table III).

Sephadex G-200 column (1.6 × 84 cm). The results of this purification procedure are summarized in Table III. Analyses of the enzymatically active sample obtained by this procedure indicated the presence of a single protein component which appeared to correspond to one of the slower moving protein components of the heat-treated S-105 preparation (Figure 3).

Comparison of the Properties of the Intestinal Promoter of Fe^{3+}–Transferrin Formation with Xanthine Oxidase. The fact that both hypoxanthine and xanthine were capable of restoring enzymatic activity to the heat-labile protein component led to an investigation of the possibility that the intestinal promoter of Fe^{3+}–transferrin formation might be xanthine oxidase. Initially, a sample of authentic xanthine oxidase was tested to determine if it possessed the ability to promote the incorporation of iron into transferrin. Xanthine oxidase was found to be an effective catalyst of Fe^{3+}–transferrin formation. The specific activity of the authentic xanthine oxidase compared favorably with that of the enzyme isolated from the intestinal mucosa (Table IV).

Physical, spectral, and kinetic properties of the two were compared to ascertain if the intestinal promoter of Fe^{3+}–transferrin formation was indeed identical with xanthine oxidase. The assay system used for the analysis of the enzymatic formation of Fe^{3+}–transferrin is substantially different from that generally used for the assay of xanthine oxidase. Furthermore, some discrepancy exists concerning the physical and kinetic properties of purified xanthine oxidase. For these reasons, a sample of high purity xanthine oxidase was compared to the purified intestinal promoter of Fe^{3+}–transferrin formation in all procedures utilized for the measurement of the physical, spectral, and kinetic properties. In all the properties measured, the intestinal promoter of Fe^{3+}–transferrin formation was virtually identical with the sample of authentic xanthine oxidase (Table IV).

Effect of Catalase and Superoxide Dismutase on the Enzymatic Formation of Fe^{3+}–Transferrin. Inclusion of large quantities of catalase (1.0 mg, 32 000 units), superoxide dismutase (1.0 mg, 3000 units), or combinations of catalase and superoxide dismutase in assay mixtures of a final volume of 1.1 mL resulted in little inhibition (<20%) of the Fe^{3+}–transferrin formation catalyzed by either the intestinal enzyme or the authentic xanthine oxidase. Inclusion of this amount of superoxide dismutase in the assay mixture was more than sufficient to completely inhibit the ability of the intestinal enzyme and authentic xanthine oxidase to reduce cytochrome *c*, which indicated that any superoxide generated during the reaction was being completely destroyed by the superoxide dismutase. The amount of catalase in these assays completely inhibited the formation of Fe^{3+}–transferrin from Fe^{2+} and apotransferrin caused by amounts of H_2O_2 far in excess of the amount of H_2O_2 that could be generated in the normal assay mixture by the intestinal enzyme or the authentic xanthine oxidase.

Discussion

The intestinal absorption of dietary iron involves three phases that can be separated from one another: (1) uptake into the mucosal cell; (2) transfer through the cytosol of the mucosal cell; (3) release from the mucosal cell to the blood plasma. Linder & Munro (1977) recently proposed that the third phase of iron absorption entails transport of a part of the iron of the mucosal cell across the serosal membrane by a process that does not require energy and involves attachment of iron to transferrin. Others (Huebers et al., 1971; Worwood & Jacobs, 1971; Pollack et al., 1972) have suggested that transferrin may exist in the mucosal cell and acquire iron. In any event, the iron that traverses the mucosal cell must eventually be incorporated into transferrin for transport in the blood plasma.

Convincing evidence (Osaki et al., 1966, 1970; Osaki & Johnson, 1969; Ragan et al., 1969; Roeser et al., 1970; Evans & Abraham, 1973; Williams et al., 1974) has established that ceruloplasmin facilitates the mobilization of iron from liver stores. Considerable evidence (Osaki et al., 1966, 1970, 1971; Osaki, 1966; Osaki & Johnson, 1969) also suggests that the iron-mobilizing activity of ceruloplasmin is the result of its ability to promote Fe^{3+}–transferrin formation. Although a physiological role for ceruloplasmin in iron mobilization has been established, Brittin & Chee (1969) found no relationship between ceruloplasmin and the intestinal absorption of iron. This led to an investigation of homogenates of intestinal mucosa to determine if an enzyme distinct from ceruloplasmin might be present which could promote Fe^{3+}–transferrin for-

Table IV: Comparison of the Purified Intestinal Promoter of Fe^{3+}-Transferrin Formation and Xanthine Oxidase

property[a]	intestinal enzyme	xanthine oxidase
mol wt (daltons)	3.44×10^5	3.44×10^5
electrophoretic mobility (mm)	12	12
A_{280}/A_{450}	6.73	7.62
fluorescence emission (nm)	515	515
sp act. [μM Fe^{3+}-Tf min^{-1} (mg of protein)$^{-1}$]	3660	3505
K_m for Fe^{2+} (μM)	46.2	49.1
K_m for hypoxanthine (μM)	6.94	6.06
K_m for xanthine (μM)	5.33	4.98
inhibition by allopurinol	>85%	>85%
superoxide formation	yes	yes
uric acid formation	yes	yes

[a] For the detailed description of the procedure utilized in the determination of each property, see Methods.

mation. The identification of such a nonceruloplasmin enzyme has been recently reported (Topham, 1978), and it has been proposed that this intestinal enzyme could serve a similar function in iron absorption as ceruloplasmin serves in iron mobilization.

The data present in this paper establish that the enzymatic promoter of Fe^{3+}-transferrin formation located in the intestinal mucosa is composed of a small, heat-stable component and heat-labile protein component. The heat-labile protein component has been identified as xanthine oxidase, and the small, heat-stable component, which is essential for activity, is xanthine which is endogenous to the intestinal homogenates. The endogenous level of xanthine in the S-105 fraction of intestinal homogenates is sufficient to yield appreciable rates of Fe^{3+}-transferrin formation without the addition of exogenous xanthine. Not until the endogenous xanthine is removed by gel filtration or ultrafiltration of the S-105 fraction does the enzymatic system become dependent upon addition of exogenous xanthine or hypoxanthine. The K_m values determined for xanthine and hypoxanthine were 5–7 μM in the assay system for Fe^{3+}-transferrin formation. In previous studies (Krenitsky et al., 1972), the K_m values obtained for xanthine and hypoxanthine for xanthine oxidase were in this order of magnitude. Thus, very little xanthine or hypoxanthine would be required to provide substantial rates of xanthine oxidase catalyzed Fe^{3+}-transferrin formation.

With the assumption of a molecular weight of 344 000, the molecular activity for the purified intestinal enzyme and for authentic xanthine oxidase is approximately 500 000 μM Fe^{3+}-transferrin min^{-1} (μM enzyme)$^{-1}$. This value is almost 3 orders of magnitude larger than the molecular activity [550 μM Fe^{3+}-transferrin min^{-1} (μM enzyme)$^{-1}$] reported for ceruloplasmin (Osaki, 1966). Ceruloplasmin exhibits two K_m values for Fe^{2+} of 0.6 and 50 μM whereas the purified intestinal enzyme and the sample of authentic xanthine oxidase exhibited a single K_m for Fe^{2+} of 46–49 μM. Thus, kinetically, intestinal xanthine oxidase compares very favorably with ceruloplasmin as an effective promoter of the incorporation of iron into transferrin.

Previous studies (Cheney & Finch, 1960; Faelli & Esposito, 1970) have demonstrated that inosine, hypoxanthine, and xanthine enhance intestinal iron absorption in vivo and in vitro. It is possible that the enhanced intestinal iron absorption observed upon administration of these compounds could be related to an increased rate of Fe^{3+}-transferrin formation catalyzed by intestinal xanthine oxidase. This would result in a greater fraction of mucosal iron being packaged in the proper form for transport in the blood stream.

The results with catalase and superoxide dismutase strongly suggest that the formation of Fe^{3+}-transferrin from apotransferrin and Fe^{2+} which is promoted by the intestinal enzyme and authentic xanthine oxidase is not simply the result of the generation of reactive forms of oxygen (H_2O_2 or O_2^-) by these enzymes that would in turn oxidize iron. Additional studies concerning the mechanism by which intestinal xanthine oxidase promotes the incorporation of iron into transferrin and the possible physiological significance of the activity are currently under investigation.

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