Identification and Purification of a Non-Ceruloplasmin Ferroxidase of Human Serum*

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SUMMARY

A non-ceruloplasmin ferroxidase (ferroxidase-II) protein was isolated from human serum and completely resolved from ceruloplasmin by DEAE-Sephadex A-50 chromatography. This protein was isolated and purified approximately 500-fold by utilization of the Cohn IV-1 fraction, DEAE-Sephadex A-50 chromatography, and gel filtration on Agarose A-15m, followed by gel filtration on Agarose A-50m. Ferroxidase-II differed from ceruloplasmin in many respects. Three of the most important differences were that ferroxidase-II: (a) was yellow rather than blue as ceruloplasmin, (b) it was not inhibited by azide, and (c) it exhibited no p-phenylenediamine oxidase activity. Ferroxidase-II also differed significantly from the known plasma amine oxidases. The ferroxidase activity of ferroxidase-II was not lost by dialysis or ultrafiltration, but was inactivated by heat treatment and was proportional to the protein concentration at all stages of purification. Oxygen consumption with ferroxidase-II was observed simultaneously with iron oxidation.

The ferroxidase-II activity in Wilson's disease serum represented a much larger percentage of the total ferroxidase activity than in normal serum. Although ferroxidase-II was decreased in Wilson's disease serum, it was reduced to a lesser extent than the ceruloplasmin ferroxidase activity. Thus ferroxidase-II may account for the lack of correlation of ferroxidase activity with p-phenylenediamine oxidase activity of Wilson's disease serum and may be responsible for the maintenance of near normal iron metabolism despite the low levels of ceruloplasmin.

The catalysis of the oxidation of Fe(II) to Fe(III) by ceruloplasmin results in an increased rate of formation of transferrin from apotransferrin (1-3). This observation led to the proposal for a biological role of ceruloplasmin in promoting the rate of iron saturation of apotransferrin and in stimulating iron utilization (4). Later Osaki and Johnson (5), Osaki et al. (6), Frieden and Osaki (7), and Ragan et al. (8), provided strong evidence obtained from both systems in vivo and in vitro which supported the idea that ceruloplasmin functions as a serum ferroxidase in promoting Fe(III)-transferrin formation.

If iron metabolism is dependent on ceruloplasmin, we might expect a disturbance in iron metabolism in Wilson's disease, a disorder characterized by low plasma ceruloplasmin and the accumulation of copper in the liver and brain. However, most Wilson's disease subjects have been found to have low normal or normal levels of iron transport (7). In attempting to correlate the aryldiamine oxidase activity with the ferroxidase activity of sera from Wilson's disease patients, it was observed that these sera had more ferroxidase than expected (7, 9). The oxidation of Fe(II) by serum components other than ceruloplasmin has been previously observed (10, 11). Lee et al. (11) have proposed citrate as an alternative source of ferrous iron-oxidizing activity in low ceruloplasmin serum. Frieden and Osaki (7) suggested the possibility of an alternative protein ferroxidase, quite different from ceruloplasmin, which might substitute for the normal enzyme in Wilson's disease serum.

The present paper describes the identification and isolation of an alternative protein ferroxidase of normal human serum which differs greatly from ceruloplasmin. The isolation, purification, and partial characterization of this non-ceruloplasmin ferroxidase (ferroxidase-II) protein from the Cohn IV-I fraction of serum is described.

EXPERIMENTAL PROCEDURE

Materials

Serum—Fresh samples of 5 ml of whole blood from many donors were collected and allowed to clot. The serum was separated by centrifugation, stored at 4°, and used within several hours. Alternatively, outdated blood was obtained from the Leon County Blood Bank, Tallahassee, Florida. The plasma was separated by centrifugation and the citrate which had been added to prevent clotting was removed by dialysis against 0.1 M acetate buffer, pH 6.0. Any precipitate formed during the dialysis procedure was removed by centrifugation. These samples had essentially the same ferroxidase-II activity as fresh serum samples.

Cohn Fractions of Human Serum—Small quantities of Cohn fractions (II, III, III-0, IV-1, IV-4, V) in lyophilized form were obtained for preliminary studies from Nutritional Biochemicals. A large quantity (10 kg) of Cohn IV-I fraction in lyophilized form was obtained from the E. R. Squibb and Sons, Inc., New York, New York, through an arrangement with Dr. G. Jamieson

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of The American National Red Cross, Blood Research Laboratories, Bethesda, Maryland, 20014. Extensive purification of the ferroxidase-II protein was performed from this material.

**Ferroin Iron**—Crystalline ferrous ammonium sulfate hexahydrate [Fe(NH$_4$)$_2$(SO$_4$)$_2$·6 H$_2$O; J. T. Baker Chemical Company, Lot 25890] was dissolved in distilled, doubly deionized water to a concentration of 1 × 10$^{-4}$ M, and used as substrate.

**Apotransferrin**—A 2.0% (w/v) solution of iron-free transferrin (apotransferrin, Hoechst Company, Cincinnati, Ohio) was prepared in distilled, doubly deionized water and extensively dialyzed as previously recommended (12).

**Chromatographic Materials**—DEAE-Sephadex A-50 (Pharmacia) was prepared with acetate as the counter ion and was equilibrated with 0.05 M acetate buffer, pH 5.5. Agarose A-15m and Agarose A-50m (Calbiochem), obtained in fully hydrated form, were equilibrated with 0.05 M acetate buffer, pH 5.5, prior to use.

**Methods**

**Enzymic Assays**—The ferroxidase assays were carried out spectrophotometrically in a manner similar to that previously described (9). Quartz spectrophotometric cuvettes, 1.5 ml in capacity and 1 cm in path length, contained 0.340 ml of a 0.6 N hydrogen peroxide solution (59.0 g of 30% hydrogen peroxide in 1 liter) and 0.360 ml of a 5.0 M potassium phosphate buffer, pH 6.0. The reaction was initiated by the addition of substrate. No difference in the rate of Fe(II) oxidation was observed when 1.0 ml of a solution of Fe(II) concentration was 30 μM. The time course of absorbance change at 460 nm resulting from the formation of transferrin from apotransferrin and Fe(III) was recorded with a Cary 15 spectrophotometer equipped with a 0.1 absorbance scale and a constant temperature cell at 30.0 ± 0.1°C. A control containing no enzyme solution was included with each assay to determine the nonenzymic rate of Fe(II) oxidation in the reaction mixture. This value was always subtracted from the observed enzymic rate to obtain the true enzymic rate. In all assays, the value for the nonenzymic rate of iron oxidation was less than 5% of the enzymic rate.

Since 1.0 mM azide inhibits ceruloplasmin more than 98% and does not inhibit ferroxidase-II (see "Results"), the ferroxidase activity of each component of human serum and the Cohn IV-1 extract could be determined by conducting the ferroxidase assays in the presence and absence of 1 mM azide. Two microliters of a 0.50 M NaNO$_3$ solution were added prior to the addition of the enzyme preparation to the reaction mixture. Alternatively, the enzyme preparation and all other components of the reaction mixture except substrate, Fe(II), were initially incubated for 5 min with azide. The reaction was initiated by the addition of substrate. No difference in the rate of Fe(II) oxidation was observed with either order of addition.

**p-Phenylenediamine oxidase assays** were carried out as previously described (2, 4).

**Disc Gel and Immunoelectrophoresis**—Disc gel electrophoresis was done with a 3% large pore polyacrylamide gel as previously described (13). The gels were subjected to 3 ma per gel for 90 min at pH 8.8, 0.025 M Tris glycine buffer. After electrophoresis the gels were stained with Coomassie Blue as described by Chrambach et al. (14).

Immunoelectrophoresis was carried out as previously described (15) except for one modification. The electrophoresis was continued for 2 hours at 45 volts at 4°C. Slides (75 × 25 mm) were stained with buffer black or oil red, a stain for lipoproteins (15).

**Identification and Isolation of Ferroxidase-II from Human Serum**

**RESULTS**

**Identification of non-ceruloplasmin ferroxidase in normal human serum and Wilson's disease serum**

Serum samples were obtained as described under "Materials." Dialyzed sera were prepared by dialysis for 24 hours at 4°C with several changes of the designated buffer. Wilson's disease sera, in the frozen state, were generously donated by Dr. G. Fleisher, Mayo Clinic, Rochester, Minnesota, and Dr. A. Morrell, Einstein Medical College, New York. Enzymic assays and protein determinations were carried out as described under "Methods."

**Table I**

| Sample        | Specific activity (μM/min/mg protein) | Percentage of total ferroxidase caused by ferroxidase-II | Specific activity, no azide (μM/min/mg protein) |
|---------------|-------------------------------------|--------------------------------------------------------|-----------------------------------------------|
| Normal human serum undialyzed    | 0.011                               | 6.7                                                   | 0.003                                         |
| Wilson's disease serum sample 2-502-192 | 0.003                               | 30.0                                                  | 0.003                                         |
| Wilson's disease serum sample 2-417-808 | 0.003                               | 33.0                                                  | 0.003                                         |

a Values represent the average of five assays with fresh sera from five different donors prepared as described under "Methods.

Human serum, previously equilibrated to pH 5.5 by dialysis against 0.05 M acetate buffer, was chromatographed on DEAE-Sephadex A-50 and eluted with a stepwise gradient of sodium.
serum from I)EAE-Sephadex A-50. A column (2.0 X 10 cm) of
by the ceruloplasmin-containing fractions was virtually com-
pletely ( >98%) inhibited by 1 mM azide. Thus the two fer-
roxidase proteins were completely resolved by DEAE-Sephadex
A-50 chromatography of human serum and were further distin-
guished by the inability of azide to inhibit ferroxidase-II.

Examination of Various Cohn Fractions of Human Serum for Ferroxidase-II Activity

Various lyophilized Cohn fractions of human serum were ex-
mained to determine whether the ferroxidase-II activity was
present (Table II). Two fractions contained this activity,
Cohn IV-1 and Cohn III-0. No loss of ferroxidase-II activity
from either of these fractions was observed on extensive dialysis
against 0.10 M acetate buffer, pH 6.0. Cohn IV-1 contained the
 greatest percentage of the ferroxidase-II activity relative to the
total ferroxidase activity and was used as the starting material
for the purification of ferroxidase-II.

Isolation and Purification of Ferroxidase-II from Cohn IV-1 Fraction

Lyophilized Cohn IV-1 powder (6 g) was extracted with 60 ml
of 0.05 M acetate buffer, pH 5.5, for 4 hours at 4°C with stirring.
The resulting extract was centrifuged at 25,000 × g for 30 min
to remove any undissolved materials. The supernatant from
this centrifugation was decanted carefully and dialyzed in the
cold for 24 hours with a large volume of 0.05 M acetate buffer,
pH 5.5. The buffer was changed several times during the
course of the dialysis. The dialyzed Cohn IV-1 extract was re-
centrifuged (25,000 × g, 30 min) to remove any material which
precipitated during the dialysis. The Cohn IV-1 fractionation
and lyophilization apparently resulted in a 70-fold purification
of ferroxidase-II as compared to whole human serum (Table III).
Of this extract, 2 ml were chromatographed on a DEAE-
Sephadex A-50 column (1.5 × 6.5 cm). The elution pattern
of the ferroxidase activities was identical with that of fresh
whole human serum. The ferroxidase-II protein was eluted
immediately following the void volume with 0.05 M acetate buffer,
pH 5.5, containing no sodium chloride. The ferroxidase activity
of this component from Cohn IV-1 also was not inhibited by 1 mM
azide. Ceruloplasmin activity began to appear in fractions
containing 0.2 M NaCl in the acetate buffer; however the major
portion was found in the fractions containing 0.3 M NaCl.
Ceruloplasmin-containing fractions were inhibited by more than
98% with 1 mM azide. Thus the two ferroxidase proteins of the
Cohn IV-1 fraction of serum could be completely resolved when
chromatographed on DEAE-Sephadex A-50 just as with fresh
human serum.

For preparative work, the entire dialyzed extract of Cohn IV-1
(approximately 45 ml following the final centrifugation) was
chromatographed on a DEAE-Sephadex A-50 column (4.5 × 8.5
cm) with an identical elution pattern of protein and ferroxidase
activity. All the ferroxidase-II activity was eluted in the first 10
10-ml fractions, 100 ml of the 0.05 M acetate buffer, pH 5.5,
containing no NaCl. The ceruloplasmin remained as a well
defined blue-green band at the very top of the preparative
DEAE-Sephadex A-50 column until the sodium chloride concentra-
tion in the acetate buffer was increased to 0.2 M.

Combining the 10 fractions containing ferroxidase-II following
DEAE-Sephadex A-50 chromatography gave a turbid yellow
solution representing 95% recovery and a 3-fold further purification
(Table III). At this stage the combined preparation (100
ml) was concentrated to a volume of 30 ml without loss of fer-
roxidase-II activity, either by dialysis against Sephadex

Table II

| Human plasma fractions | Ceruloplasmin ferroxidase | Ferroxidase-II (±1 mM azide) | Percentage of total ferroxidase caused by ferroxidase-II |
|------------------------|---------------------------|-----------------------------|----------------------------------------------------------|
| α-Globulin Fraction IV-1 | +                         | +                           | 48.7                                                     |
| β-Lipoprotein Fraction III-0 | +                        | +                           | 9.7                                                      |
| α-Globulin Fraction IV-4+... | -                        | +                           | 0                                                        |

a Cohn fractions III (β-globulin); II (γ-globulin); and V (albumin) were also tested. None of these fractions contained either ferroxidase activity.

chloride (Fig. 1). Ferroxidase-II protein was eluted from the
column immediately following the void volume with 0.05 M
acetate buffer, pH 5.5, containing no sodium chloride. This
ferroxidase-II protein showed no difference in activity when
tested in the presence or absence of 1 mM azide. No ceruloplas-
min was eluted from the column until the sodium chloride concentra-
tion in the buffer was increased to 0.2 M. Most of the
ceruloplasmin was eluted when the sodium chloride concentration
was increased to 0.3 M, a result typically observed in the prepara-
tion of ceruloplasmin (17). The ferroxidase activity exhibited
by the ceruloplasmin-containing fractions was virtually com-
pletely (>98%) inhibited by 1 mM azide. Thus the two fer-

![Figure 1](image-url)
TABLE III
Isolation and purification of ferroxidase-II

Enzymic activities and protein concentrations were determined as described under "Methods." All values represent the average of two separate experiments in which two separate Cohn IV-1 extracts were carried through the entire purification procedure.

| Step | Total protein (mg) | Total activity, +1 μm wide AAa/10 min | Total activity, no azide AAa/10 min | Specific activity of ferroxidase-II | Fold purification | Percentage of recovery |
|------|-------------------|----------------------------------------|-------------------------------------|-------------------------------------|------------------|------------------------|
|       |                   | ΔA440/10 min/mg | ΔA440/10 min/mg | ΔA440/10 min/mg/mg protein |                  |                        |
| Whole human serum* |                 |                          |                          |                          |                  |                        |
| Crude extract of lyophilized Cohn IV-1 | 3540 | 2600 | 2270 | 0.760 | 71.6 | 100 |
| DEAE-Sephadex A-50 | 1140 | 2540 | 2350 | 2.22 | 211 | 94.5 |
| Band I, Agarose A-15m | 58.3 | 313 | 313 | 5.38 | 514 | 11.6 |
| Band II, Agarose A-15m | 517 | 2350 | 2350 | 4.54 | 423 | 87.3 |
| Band II, rechromatographed on Agarose A-50m | 294 | 1450 | 1450 | 4.93 | 470 | 54.0 |

*Value represents an average of the ferroxidase-II specific activities of five serum samples of five different donors.

Fig. 2. Elution of protein and ferroxidase activity from a column of Agarose A-15m. A column (4.5 × 53 cm) was packed with previously swollen Agarose A-15m and equilibrated with 0.05 M acetate buffer, pH 5.5. The 20 ml (970 mg of protein) of the ferroxidase-II preparation which had been concentrated following the purification with DEAE-Sephadex A-50 was applied to this column. Protein was eluted from the column with 0.05 M acetate buffer, pH 5.5. Fractions of 13 ml were collected. Ferroxidase activities (with 50 μl from each fraction) in the presence and absence of 1 mM azide and protein concentrations were determined as in Fig. 1.

G-200 or by ultrafiltration. This concentrated ferroxidase-II solution was applied to an Agarose A-15m column (4.5 × 53 cm). Protein was eluted with 0.05 M acetate buffer, pH 5.5. Fractions of 13 ml were collected. Two bands of ferroxidase-II activity were eluted (Fig. 2). The first of these bands, containing 10 to 12% of ferroxidase-II, (Band I, Fig. 2) was yellowish and very turbid and was eluted immediately after the void volume. The major portion (~90%) ferroxidase-II activity was retained on this column and eluted in Fractions 24 to 46. These fractions exhibited a yellow color but were not turbid. The ferroxidase-II preparation isolated from fresh human serum by DEAE-Sephadex A-50 chromatography (Fig. 1) was also applied to the column of Agarose A-15m. A single band of ferroxidase-II was observed with a mobility identical with the second major band shown in Fig. 2 (Band II). Thus, the first minor band of ferroxidase-II activity (Band I, Fig. 2) probably represented suspended but not truly soluble material. This aggregate may represent partially denatured, insoluble, or lipid-occluded, and still active ferroxidase-II, which may have resulted during the Cohn fractionation or lyophilisation procedure.

Fractions 26 to 42 (Band II, Fig. 2) from the Agarose A-15m column were combined. The combined fractions (~200 ml) represented an 87.3% recovery of the ferroxidase-II activity and a further purification of 2-fold (Table III). Again 1 mM azide did not inhibit the ferroxidase-II preparation. This preparation was concentrated to a final volume of 30 ml by either desiccation against Sephadex G-200 or by ultrafiltration. Neither procedure resulted in a loss of ferroxidase-II activity. Concentrated Band II was divided into two equal 15-ml portions. Each portion was chromatographed on a column of Agarose A-50m (3 × 45 cm). A single band of ferroxidase-II activity was obtained (Fig. 3), which eluted with the major protein band.

Fig. 3. Gel filtration of the Band II (Fig. 2) of ferroxidase-II isolated from the Agarose A-15m column on a column of Agarose A-50m. A column (3 × 45 cm) of previously swollen Agarose A-50m, equilibrated with 0.05 M acetate buffer, pH 5.5, was prepared. A sample of 15 ml (135 mg of protein) which had been concentrated following isolation by Agarose A-15m gel filtration was applied to this column. Protein was eluted with 0.05 M acetate buffer, pH 5.5. Fractions of 12 ml were collected. Ferroxidase activities (with 50 μl from each fraction) in the presence and absence of 1 mM azide and protein concentrations were determined as in Fig. 1.
A minor, more slowly moving band of protein with no ferroxidase activity was also observed. Fractions 15 to 22 (Fig. 3) from this column were combined yielding a 54% recovery of ferroxidase-II and a further small purification. Again, no inhibition of the ferroxidase activity of this preparation was observed with 1 mM azide. The total purification procedure routinely yielded ferroxidase-II of 400- to 600-fold purification (Table III). At the final stage of purification the enzyme was stable when stored at 4° for at least 2 weeks. After this time period, some slow precipitation and loss of activity was observed.

Properties of Purified Ferroxidase-II

Disc Gel and Immunoelectrophoresis of Ferroxidase-II after Final Purification Step—When the purified ferroxidase-II preparation was subjected to gel filtration on a column of Sephadex G-200 (2.5 × 45 cm), all of the ferroxidase activity was excluded from the column and eluted immediately following the void volume. However, the same preparation was retained on Agarose A-15m. This indicated that the protein must have a large molecular weight, probably greater than 800,000, the exclusion limit of Sephadex G-200 for globular proteins. Accordingly, for disc gel electrophoresis, a 3.1% large pore polyacrylamide gel was chosen. Electrophoresis of purified ferroxidase-II indicated a single major band of protein which migrated approximately one-half the distance of the tracking dye (Fig. 4). The maximum molecular weight a protein can possess and still migrate into this gel is approximately 2,000,000 (18). Thus from the gel filtration data and the disc gel electrophoresis it would appear that ferroxidase-II must have a molecular weight between 800,000 to 2,000,000, which is much larger than the value of 100,000 for ceruloplasmin (19).

The purified ferroxidase-II was also subjected to immunoelectrophoresis. A single major precipitation arc was observed when cross-reacted with whole anti-human serum and stained with buffalo black (Fig. 4) or oil red, a stain for lipoproteins (15). The ferroxidase-II protein migrated only a small distance toward the anode and it has a mobility similar to that of serum lipoproteins. Ceruloplasmin when subjected to immunoelectrophoresis under the same conditions migrated much further toward the anode than the ferroxidase-II protein. The electrophoretic data suggest that the combination of the previously mentioned fractions from the Agarose A-50m column yields a relatively homogeneous ferroxidase-II protein.

Further Evidence for Enzymic Nature of Ferroxidase-II—Several facts previously mentioned all point to the macromolecular nature of ferroxidase-II. Extensive dialysis and concentration by ultrafiltration did not result in any loss of ferroxidase-II activity. Ferroxidase-II was purified by chromatographic methods usually employed for soluble proteins. Additional data also indicated the enzymic nature of the reaction catalyzed by ferroxidase-II. A linear relationship was observed between the initial velocity of the ferroxidase reaction and the concentration of ferroxidase-II protein in the reaction mixture (Fig. 5A).
the purified protein. Ferroxidase-II was inactivated by heat treatment (Fig. 5B) and partial inactivation was noted earlier on prolonged storage (>2 weeks) at 4°.

The specific activity of purified ferroxidase-II in units of ΔΔAbs per 10 min per mg of protein was 4.9. The specific activity of purified ceruloplasmin in these same units is 30. However, if the approximate molecular weights of each ferroxidase are taken into consideration, the molar activities are similar. Another indication of the enzymic "oxidase" nature of the ferroxidase-II protein was the fact that oxygen consumption was observed simultaneously with Fe(II) oxidation measured as transferrin protein.

**Substrate Specificity**—p-Phenylenediamine was tested as a possible substrate for ferroxidase-II as described under "Methods." Ferroxidase-II differed from ceruloplasmin in substrate specificity in that it had no p-phenylenediamine oxidase activity (12, 20). Another copper-containing plasma enzyme which could possibly serve as the non-ceruloplasma ferroxidase is plasma monoamine oxidase. However, ferroxidase-II did not catalyze the oxidation of benzylamine, a good substrate for human plasma monoamine oxidase (21). Two potent inhibitors of plasma monoamine oxidase, N-(3,4-dichlorophenacyl)cyclopropylamine hydrobromide and N-[β-(2-chlorophenoxy)ethyl]cyclopropylamine chloride (Eli Lilly and Company, Indianapolis, Indiana) (22) did not inhibit Fe(II) oxidation by ferroxidase-II.

**Visible Absorption Spectrum of Purified Ferroxidase-II**—The purified ferroxidase-II exhibited a distinct absorbance maximum at 402 nm (Fig. 6). Shoulders on this absorbance maximum were observed at 448 and 482 nm. This visible absorbance spectrum was quite different from that of ceruloplasmin which has absorbance maxima at 794 nm (850), 610 nm (4400), 450 nm (460), and 332 nm (1600) (23). The purified ferroxidase-II showed none of the intense blue color (610-nm absorption) which is characteristic of ceruloplasmin.

The 402-nm maximum was shifted in the presence of 1 mM azide to a 415-nm maximum; however, the shoulders at 448 and 482 nm were unaffected. When the visible spectrum was recorded immediately following the addition of Fe(II) to the enzyme solution, the absorbance at 402 nm was significantly reduced and the shoulders at 448 and 482 nm disappear. When the spectrum was recorded immediately after the addition of Fe(II) to the enzyme solution containing 1 mM azide, the 415-nm peak was significantly reduced and the shoulders at 448 and 482 nm again disappeared. The iron oxidase activity of ferroxidase-II thus correlated with reduction of its characteristic visible absorbance spectrum. Azide apparently affected the chromophore responsible for the 402-nm absorbance by a shift of this maximum to 415 nm; however, the azide did not block reduction of the visible absorbance when Fe(II) was added.

**Copper Content of Purified Ferroxidase-II**—Copper remained firmly associated with ferroxidase-II throughout the purification procedure. Chemical analyses performed by the method of Wharton and Radin (24) on ferroxidase-II after the final step of purification and passage over a Chelex 100 column to remove nonprotein-bound copper, indicated a copper content of 12 μmoles of copper per mg of protein. These same enzyme preparations exhibited a typical electron paramagnetic resonance signal (Fig. 7) with a g value very close to that observed for many other copper-containing proteins (25). This tightly bound copper may be responsible at least in part, for the visible absorption spectrum of the purified ferroxidase-II.

**DISCUSSION**

The properties which distinguish ferroxidase-II from ceruloplasmin are summarized in Table IV. The first four of these

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2 R. W. Topham and D. A. Johnson, unpublished data.

3 Gram atomic absorption coefficients in parentheses (22).
represent important kinetic differences. Particularly interesting was the fact that ferrosidase-II had no aryldiamine oxidase activity. The specific ferroxidase activities of ceruloplasmin and ferroxidase-II, expressed on a milligrams of protein basis, differ by a factor of 6. However, if the respective molecular weight of each ferroxidase is taken into consideration the molar activities are very close. The specific activities per copper atom are also similar. The last six properties represent the important chemical differences. It is interesting to note that the immunoelectrophoretic mobility, the oil red-staining property, and the large molecular weight suggest that ferroxidase-II may be a serum lipoprotein (present paper and Reference 10). Ceruloplasmin is approximately 0.28% copper; however ferroxidase-II contained approximately 0.08% copper. The copper content of ferroxidase-II is similar to several other copper proteins, e.g. tyrosinase and cytochrome oxidase (24).

Similarly, many properties distinguish ferroxidase-II from human plasma monoamine oxidase. (a) Ferroxidase-II did not catalyze the oxidation of benzylamine. (b) No inhibition of ferroxidase-II was observed with potent inhibitors of plasma monoamine oxidase. (c) The molecular weights of the well characterized plasma monoamine oxidases are 900,000 (bovine plasma enzyme) and 190,000 (pig plasma enzyme) (26). Ferroxidase-II appears to have a much larger molecular weight. (d) Purified human plasma monoamine oxidase preparations were colorless (21) and purified bovine plasma monoamine oxidase preparations were pink (27). Ferroxidase-II preparations are yellow in appearance. (e) The visible spectral features of purified ferroxidase-II preparations correlated with their ferroxidase activity. No correlation between the visible spectral features and the monoamine oxidase activity was observed for purified human plasma monoamine oxidase preparations (21).

The iron oxidation reaction catalyzed by ferroxidase-II appears to be an "enzymic oxidase" reaction for the following reasons. (a) The initial velocity of the ferroxidase-II reaction was directly proportional to the concentration of protein at all stages of purification. (b) Inactivation of these ferroxidase-II preparations was achieved by heat treatment and with the purified material after prolonged storage in the cold. (c) The molar activity of ferroxidase-II or specific activity per copper atom is close to that of ceruloplasmin although the specific activities on a milligrams of protein basis were quite different. (d) Oxygen consumption was observed simultaneously with the catalysis of iron oxidation by the ferroxidase-II. (e) With regard to substrates thus far tested, ferroxidase-II apparently exhibits specificity for Fe(II).

One major objection has been raised to the proposal that the physiological function of ceruloplasmin in blood is to catalyze the oxidation of Fe(II) in plasma to Fe(III), promoting the iron saturation of transferrin, and thereby stimulating the over-all turnover of iron from tissue depots. This objection stems from the fact that there appears to be little disturbance in iron metabolism in Wilson's disease, a disorder characterized by low plasma ceruloplasmin. The finding that ferroxidase-II exists in human serum may resolve this apparent paradox. Our results indicate that while the ceruloplasmin level of Wilson's disease serum decreases very dramatically, ferroxidase-II is less affected. The total ferroxidase activity of Wilson's disease sera is approximately 5 to 10% of that of normal sera (see "Results" and Reference 7). Otsuki et al. (6) have found that only about 10% of the total ferroxidase activity of normal serum was necessary in order to observe maximum iron mobilization response from liver. Thus the total ferroxidase of Wilson's disease serum would be enough for normal or low normal iron metabolism. However, without ferroxidase-II which accounts for 30% of the total ferroxidase in Wilson's disease serum, this would not be possible. Ferroxidase-II also accounts for the fact that p-phenylenediamine oxidase activity did not correlate with ferroxidase activity in Wilson's disease sera and in sera from some other animals (6, 7, 9).

Further investigations with ferroxidase-II concerned with the kinetics of the iron oxidation and oxygen consumption reactions, the possible role of copper in the enzymic reaction, the physical-chemical properties, the substrate specificity, and the physiological significance in Wilson's disease and other species are currently in progress.

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