Isolation from Bovine Spleen of a Green Heme Protein with Properties of Myeloperoxidase*

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A novel green heme protein from bovine spleen has been purified to apparent homogeneity. The visible spectrum, with unusually long wavelength absorptions due to α-, β-, and γ-porphyrin bands, the EPR g values of 6.81, 4.99, and 1.84, and the peroxidase activity are similar to those of myeloperoxidase (EC 1.11.1.7). The observed molecular mass of 97,000 daltons (established by gel permeation chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis studies) clearly distinguishes it from myeloperoxidase, as does the observed substrate specificity (e.g. oxidation of iodide but not ascorbate). Optical spectra of ferric and ferrous forms of the enzyme in the native state, complexed with ligands (CN-, CO, NO, N3-) and as the NO adduct were run using an argon flushed cell; all reduced samples were washed twice with 100 ml of 0.2 M KCl, 0.05 M sodium acetate, pH 5.0, and eluted with 0.05-1.0 M KCl gradient in 0.05 M sodium acetate buffer, pH 5.0. The KCl concentration was monitored by measuring the conductivity of the samples and comparing to a set of KCl/buffer standards. The fractions with absorbance ≥ 0.05 at 434 nm were pooled, concentrated to 5 ml by ultrafiltration (Amicon 8MC, PM-10 membrane), loaded onto a Sephadex G-75 column (1.5 × 85 cm), and eluted with 0.2 M KCl. The green fractions with A326/A434 ≥ 0.6 were combined and concentrated to ~1 ml as before.

The final purification step was rechromatography on CM-52 carboxymethyl-cellulose. After loading the green heme protein as before and eluting with 100 ml of 0.2 M KCl, 0.05 M sodium acetate, pH 5.0, the column was sectioned, and the first 5 mm were placed in 5.0 ml of 2.0 M KCl and allowed to stand for 20 min. The suspension was then centrifuged briefly and the purified green heme protein was decanted.

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solution of dithioerythritol. The pyridine hemochromogens were prepared by making the enzyme solution 30% (v/v) in pyridine. Some of the optical spectral data were obtained on samples of protein prior to the CM-52 column sectioning; electrophoresis results indicated that these samples were ~80% pure, with ~20% contamination by a co-purifying purple iron-containing acid phosphatase. The latter has been purified to homogeneity by a process involving selective irreversible absorption of the green heme protein to hydroxyapatite and has separately been shown to be EPR-inactive, to have zero peroxidase activity, and to make only an insignificant (~5%) contribution to the visible absorption under the conditions examined here (10).

RESULTS

Isolation—The isolation of the green heme protein is detailed above under "Experimental Procedures." Fig. 1 shows a typical carboxymethyl-cellulose chromatogram with the KCl gradient superimposed. The green band elutes at a KCl concentration of 0.65 M, suggesting that the protein is highly basic. Fig. 2 shows the elution profile of the enzyme on Sephadex G-75; a small amount of a lower molecular weight protein elutes shortly after the green heme protein. Typical purification data are shown in Table I for the cellulose phosphate, carboxymethyl-cellulose, and Sephadex G-75 steps. A convenient criterion of purity is the ratio of the absorbance at 434 nm to that at 280 nm; this rises throughout the purification to a value of ~0.80. Analysis of the gel electrophoresis results (see below) suggests that the protein is >95% pure. Fig. 3 shows densitometer traces of three native enzyme electrophoresis disc gels: plot 1 is a scan of an unstained gel at 430 nm to show the position of the green heme protein; plot 2 is a scan of an identical gel run at the same time, but stained with iodide and H$_2$O$_2$ at pH 7 to show the location of peroxidase activity; plot 3 is a scan of another identical gel, this time stained with Coomassie blue to show the position of protein bands. The coincidence of these peaks strongly suggests that the visible absorption and peroxidase activity are due to the same apparently homogeneous protein.

Visible Spectral Data—Fig. 4 shows the spectrum of the native (Fe$^{II}$) form of the protein along with those of the CN-, N$_3$-, NO, and CO adducts of the reduced (Fe$^{II}$) form. Table II summarizes the spectral data for the α, β, and γ bands in all four spectra. Treatment with N$_2$ (75 mM) caused no discernible changes in the native spectrum. Fig. 5 shows the spectra of the reduced heme and the CN-, N$_3$-, NO, and CO adducts of the reduced (Fe$^{II}$) form. Table II summarizes the spectral data for the various forms of the enzyme; values reported for derivatives of myeloperoxidase are included for comparison.

Electron Paramagnetic Resonance Spectroscopy—Fig. 6 shows the X-band EPR spectrum of the green heme protein. The g values of 6.81, 4.99, and 1.94 are characteristic of high

![Fig. 1](image1.png)

**Fig. 1.** Ion exchange chromatography of the green heme peroxidase on carboxymethyl-cellulose (CM-52 Whatman). Elution by KCl gradient (Δ) with absorption at 280 nm (●) and at 430 nm (○) is shown.

![Fig. 2](image2.png)

**Fig. 2.** Chromatography of the green fractions from CM-52 on Sephadex G-75 with absorption at 430 nm (—) and 280 nm (---) shown.

![Fig. 3](image3.png)

**Fig. 3.** Disc-gel electrophoresis of green heme peroxidase on 7.5% polyacrylamide. Plot 1, λ = 430 nm, no stain; plot 2, I$\text{H}_2$O$_2$, activity stain, λ = 350 nm; plot 3, Coomassie blue stain, λ = 625 nm.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Purification of green heme protein from beef spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step</td>
<td>Total protein$^a$</td>
</tr>
<tr>
<td>Acid extract$^d$</td>
<td>111,000</td>
</tr>
<tr>
<td>P-11</td>
<td>1,760</td>
</tr>
<tr>
<td>CM-52</td>
<td>60</td>
</tr>
<tr>
<td>G-75</td>
<td>21</td>
</tr>
<tr>
<td>CM-52-column sectioning</td>
<td>12</td>
</tr>
</tbody>
</table>

$^a$Estimated by assuming that an absorption at 280 nm of 1.0 is equivalent to 1.0 mg of protein/ml.

$^b$Determined by oxidation of p-aminobenzoate (0.04 M) in 0.1 M phosphate buffer, pH 7.0, with 0.5 mM H$_2$O$_2$; AAS$25$ of 0.010/min is one unit of enzyme activity.

$^c$From 1000 g of beef spleen.

$^d$May include non-green heme peroxidases.
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TABLE II

Summary of visible absorption data for green heme peroxidase and myeloperoxidase

<table>
<thead>
<tr>
<th>Form*</th>
<th>Absorption maxima of following bands (nm):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a</td>
</tr>
<tr>
<td>Native (Fe³⁺)</td>
<td>574</td>
</tr>
<tr>
<td>Native (Fe³⁺)</td>
<td>580</td>
</tr>
<tr>
<td>GHP (Fe³⁺) + CN⁻</td>
<td>632</td>
</tr>
<tr>
<td>GHP (Fe³⁺) PyHC⁺</td>
<td>580</td>
</tr>
<tr>
<td>MPO (Fe³⁺) PyHC⁺</td>
<td>590</td>
</tr>
<tr>
<td>MPO (Fe³⁺) PyHC⁺</td>
<td>590</td>
</tr>
<tr>
<td>GHP (Fe³⁺)</td>
<td>597</td>
</tr>
<tr>
<td>GHP (Fe³⁺) PyHC⁺</td>
<td>590</td>
</tr>
<tr>
<td>MPO (Fe³⁺)</td>
<td>636</td>
</tr>
<tr>
<td>MPO (Fe³⁺) PyHC⁺</td>
<td>636</td>
</tr>
<tr>
<td>GHP (Fe³⁺)+ CN⁻</td>
<td>613</td>
</tr>
<tr>
<td>MPO (Fe³⁺)+ CN⁻</td>
<td>615</td>
</tr>
<tr>
<td>GHP (Fe³⁺)+ N₂⁻</td>
<td>614</td>
</tr>
<tr>
<td>MPO (Fe³⁺)+ N₂⁻</td>
<td>615</td>
</tr>
<tr>
<td>GHP (Fe³⁺)+ CO⁺</td>
<td>629</td>
</tr>
<tr>
<td>MPO (Fe³⁺)+ CO⁺</td>
<td>634</td>
</tr>
<tr>
<td>GHP (Fe³⁺)+ NO⁺</td>
<td>626</td>
</tr>
</tbody>
</table>

*GHP, green heme protein described in this work; MPO, myeloperoxidase; PyHC, pyridine hemochromogen; sh, poorly resolved shoulder.

1. This work.
2. Ref. 5.
5. Ref. 23.

Fig. 4. Visible absorption spectra of the green heme peroxidase. Shown are the Fe³⁺ native enzyme (-----), the Fe³⁺ cyanide adduct (-----), the oxidized pyridine hemochromogen (------), and the reduced pyridine hemochromogen (. . . . ).

Fig. 5. Visible absorption spectra of the reduced (Fe²⁺) forms of the green heme peroxidase. Shown are the reduced native form (-----), the CN⁻ adduct (-----), the N₂⁻ adduct (-----), the NO adduct (-----), and the CO adduct (-----).

Spin ferric heme (for example, the fluoride adduct of catalase (11)).

Molecular Weight Studies—The similarity of the above visible spectra to those of the corresponding derivatives of myeloperoxidase (12-15) suggested that the green heme protein from beef spleen might be identical with myeloperoxidase.

Fig. 7 shows the molecular weight estimation of the green heme protein on a Sephadex G-75 column (85 x 1.5 cm) using four marker proteins of known molecular weight. A value of 57,000 is obtained for the molecular weight of the green heme protein.

Fig. 6. EPR spectrum of the green heme peroxidase at gain 1 x 10⁴ (compilation of 10 scans on signal averaging package). Conditions for EPR spectroscopy were: frequency, 9.469 GHz; microwave power, 10 milliwatts; modulation amplitude, 5 G; scanning rate, 2000 G min⁻¹; time constant, 0.2 s; modulation frequency, 100 KHz; temperature, 10.1 K.

Fig. 7. Gel permeation molecular weight estimation on G-75. Standards were: A, β-lactoglobulin; B, α-chymotrypsinogen; C, ovalbumin; D, bovine serum albumin. Vₑ (elution volume) was determined by intercept of the slope of the leading edge of each peak.
protein from beef spleen. Similar results were obtained by SDS-polyacrylamide gel electrophoresis; the data are shown in Fig. 8. Comparison of the SDS and native gel data indicate the presence of a single polypeptide chain of molecular weight 56,000. Scanning of the stained native gel on the Gilford DU showed the green heme protein to be ≥95% pure.

Iron Content—Colorimetric iron analyses suggest an iron content of ~1.2 g atoms of Fe/M, = 57,000. The protein concentration used to obtain this value was determined from the absorbance at 280 nm and the assumption that the extinction coefficient at that wavelength is the same as that reported by Agner for crystalline myeloperoxidase (15). This result must thus be regarded as approximate, until sufficient quantities of the green heme peroxidase are obtained to permit an accurate gravimetric determination of the extinction coefficient.

Substrate Specificity—Several typical substrates for peroxidases were tested at pH 5.0 and pH 8.5; the rate of reaction was monitored by visible or UV absorption change at the appropriate wavelength. Results are given in Table III. The green heme protein was also tested for catalase activity by addition of enzyme to 5 mM HzO2 solution at pH 8.5; no catalase activity, as evidenced by evolution of gas or decrease in absorption at 240 nm, was observed. Addition of the green heme protein to pyrogallol at pH 8.5 in the absence of H2O2 resulted in the slow formation of a red color, suggesting weak oxidase activity. Using the same conditions as Hosoya et al. (19) for iodide oxidation and assuming that an absorption of 1.0 at 434 nm corresponds to a protein concentration of 1.0 mg/ml, we find that the specific activity of the green heme protein or iodide oxidation is 30 units/mg. This is below the value reported for lactoperoxidase (108 units/mg), but much higher than that found for myeloperoxidase (5 units/mg) (20).

Attempts at Heme Removal—Two attempts were made to remove the heme moiety from the protein. First, the classical HCl/acetonc method of extraction was attempted. One ml of enzyme (~2 mg/ml) was mixed with 1 M HCl in acetone (2 ml). Precipitation of the protein occurred and the green color due to the heme remained associated with the precipitated protein.

The method of Paul (21) for cleaving thioethers in cytochrome c with AgNO3; in glacial acetic acid was also attempted. Again the green chromophore remained with the protein fraction, although significant bleaching of the green color occurred. Similar bleaching was also noted upon addition of a large excess of HCl (final concentration, ~1 M).

**DISCUSSION**

The isolation of the green heme protein described above relies on a convenient batch absorption to and elution from cellulose phosphate (P-11). This results in at least an 80-fold purification with very good (~70%) recovery. At this point, however, there is still too much residual absorbance in the 400- to 450-nm region for the A434/A280 ratio to give any indication of the actual amount of green heme protein present. The subsequent step, chromatography on carboxymethyl-cellulose, consistently results in only a 20% recovery of activity. This, together with the observation that a dark band appears at the top of the CM-52 column during loading and does not migrate even with 2.0 M KCl, suggests that at least a portion of the peroxidase activity in the crude extract may be associated with other colored proteins. The actual recovery of green heme protein may thus be higher than indicated in Table I; the protein has been purified at least 900-fold with ≥10% recovery in only four steps. The major impurity in the preparation after Sephadex G-75 chromatography is another iron protein, the purple acid phosphatase known to be present in beef spleen (10, 22-24). Absorption of the green protein onto a CM-52 column followed by column sectioning removes the purple acid phosphatase impurity.

A comparison of spectroscopic properties suggests that the chromophores of the green heme peroxidase and of myeloperoxidase are similar, if not identical. Thus, the position of the Soret (γ) peaks in the optical spectra of the two proteins agree within 1-2 nm for the native (ferric), oxidized, and reduced pyridine hemochromogens and ferrous cyanide and azide derivatives, while the positions of the long wavelength (α) peaks are also generally in agreement. The observed differences in α peaks are greatest for the pyridine hemochromogens (approximately 7-10 nm) and may reflect the use of different concentrations of pyridine in preparing the samples. The only serious discrepancy is in the properties of the CO complex. We observe Soret and α bands at 459 and 629 nm, respectively, compared to 468 and 634 nm for myeloperoxidase (25). In addition to the position of the peaks, the observed ratios of intensities of the long wavelength features to that of the Soret band are also essentially the same as in myeloperoxidase. These spectral features are all substantially red-shifted compared to the spectra of analogous derivatives of common heme proteins, such as cytochromes b, a, and even

**Fig. 8.** SDS-gel electrophoresis estimate of the molecular weight of the green heme peroxidase. Standards were: A, lysozyme; B, β-lactoglobulin; C, α-chymotrypsinogen; D, ovalbumin; E, bovine albumin. Electrophoretic conditions were: 1.5 mm gel thickness; 4 mA/channel; 4 °C; 15% acrylamide gel; 5% stacking gel; gel divided by distance of front from origin.

**Table III**

Substrate specificity of the green heme peroxidase from bovine spleen

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration (mM)</th>
<th>pH 5.0</th>
<th>pH 8.5</th>
<th>λ</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-</td>
<td>0.05</td>
<td>500</td>
<td>400</td>
<td>350</td>
<td>0.5 unit/mg</td>
</tr>
<tr>
<td>I-</td>
<td>0.05</td>
<td>500</td>
<td>400</td>
<td>350</td>
<td>0.5 unit/mg</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>0.005</td>
<td>500</td>
<td>400</td>
<td>350</td>
<td>No reaction</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>0.005</td>
<td>500</td>
<td>400</td>
<td>350</td>
<td>No reaction</td>
</tr>
<tr>
<td>Pyrogallol1</td>
<td>0.005</td>
<td>500</td>
<td>400</td>
<td>350</td>
<td>0.13 A/min</td>
</tr>
<tr>
<td>Pyrogallol2</td>
<td>0.005</td>
<td>500</td>
<td>400</td>
<td>350</td>
<td>0.13 A/min</td>
</tr>
<tr>
<td>1-Naphthol</td>
<td>0.035</td>
<td>500</td>
<td>400</td>
<td>350</td>
<td>No reaction</td>
</tr>
<tr>
<td>1-Naphthol</td>
<td>0.035</td>
<td>500</td>
<td>400</td>
<td>350</td>
<td>No reaction</td>
</tr>
<tr>
<td>p-Aminobenzoate</td>
<td>0.005</td>
<td>500</td>
<td>400</td>
<td>350</td>
<td>0.2 A/min</td>
</tr>
<tr>
<td>p-Aminobenzoate</td>
<td>0.005</td>
<td>500</td>
<td>400</td>
<td>350</td>
<td>0.2 A/min</td>
</tr>
<tr>
<td>Pyrogallol1</td>
<td>0.005</td>
<td>500</td>
<td>400</td>
<td>350</td>
<td>0.2 A/min</td>
</tr>
</tbody>
</table>

*Method of Ref. 11.
*Conditions the same as in the legend except [enzyme] = 1.5 µg/ml.
*Method of Ref. 16.
*Method of Ref. 17.
*Method of Ref. 18.
*Conditions the same as in the legend except for the absence of H2O2.

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sequent steps. We have, however, found that varying the degradation of the latter during the acid extraction and subto the heavy subunit of myeloperoxidase and arises from heme peroxidase approaches that of lactoperoxidase, an enzyme described in this paper to myeloperoxidase remains heme peroxidase cannot tolerate the relatively bulky and temperature the two proteins: that of myeloperoxidase must be able to oxidize ascorbate, a good substrate for myeloperoxidase, de- thermodynamic basis, inasmuch as ascorbate is much more easily oxidized than iodide. This selectivity cannot have a thermodynamic basis, inasmuch as ascorbate is much more easily oxidized than iodide. This selectivity cannot have a high spin state (28).

The similarities between the green heme protein and myeloperoxidase are not limited to spectroscopic properties. Both are highly basic proteins with moderate peroxidase activity towards a variety of substrates, and both appear to contain heme prosthetic groups tightly bound via covalent bonds involving other than ester or thioether linkages. There are, however, significant differences between the two proteins with regard to apparent molecular weight, substrate specificity and specific activity, and distribution in tissues, which strongly suggest that the two are not in fact identical.

Certainly the major difference between the two enzymes is the discrepancy in observed molecular weight. Our gel per- spective group. 

Finally, myeloperoxidase to date has not been isolated from reticuloendothelial tissue such as spleen. The recent report (8) of the isolation of myeloperoxidase from human monocytes suggests that it should be present in spleen, which contains significant amounts of monocytes in addition to lymphocytes and erythrocytes. The precise relationship of the green heme protein described in this paper to myeloperoxidase remains unclear. We cannot rule out the possibility that it corresponds to the heavy subunit of myeloperoxidase and arises from degradation of the latter during the acid extraction and sub- 

REFERENCES