The Reductive Cleavage of Myeloperoxidase in Half, Producing Enzymically Active Hemi-myeloperoxidase

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Reduction and alkylation of human myeloperoxidase under non-denaturing conditions results in the cleavage of this enzyme. Sedimentation equilibrium data is presented which shows that the molecular weight of the cleavage product (78,000 ± 2,000) is half that of the native enzyme (153,000 ± 4,000). We conclude that the cleavage product is the half-enzyme hemi-myeloperoxidase.

Hemi-myeloperoxidase retains both heme groups and contains both subunit types (M₂ = 57,500 and 14,000) in the same ratio as native myeloperoxidase. The two halves of native myeloperoxidase are apparently not dependent upon one another for peroxidatic activity, as the specific activity of the half-enzyme is the same as that of the native enzyme.

Analytical ultracentrifugation studies show native myeloperoxidase has a sedimentation coefficient of 8.0 and an axial ratio of 5:1, while hemi-myeloperoxidase has a sedimentation coefficient of 4.3 and an axial ratio of 10:1. When [³H]iodoacetate acid was used to prepare hemi-myeloperoxidase, the label incorporated with a stoichiometry of 1.2 [³H]carboxymethyl] groups per hemi-myeloperoxidase, with 90% of this label associated with the heavy subunit.

From these observations we conclude that native myeloperoxidase contains two heavy-light protomers, which are joined along their long axes by a single disulfide bond between the heavy subunits. Selective reduction of this disulfide bond by the use of non-denaturing conditions results in the formation of hemi-myeloperoxidase, a catalytically active heavy-light protomer of native myeloperoxidase.

Myeloperoxidase (donor: H₂O₂ oxidoreductase, EC 1.11.1.7) appears to be uniquely localized in mammalian neutrophils and monocytes (1). A major function of neutrophils is to kill ingested bacteria (2); a function partially fulfilled by myeloperoxidase. This enzyme has been shown to have a bactericidal effect in the presence of H₂O₂ and a halide such as chloride, iodide, or bromide (3, 4).

Canine myeloperoxidase contains four subunits; two heavy (M₂ = 57,500) and two light (M₂ = 10,500) polypeptide chains (5). Human myeloperoxidase has been reported to have subunit molecular weights of 54,000 and 14,000 (6). Dimethylsulphate cross-links the two light subunits of canine myeloperoxidase with no evidence of the cross-linking of the two heavy subunits (5). This observation led to the proposal of the quaternary structure of myeloperoxidase as a linear array of heavy-light:light:heavy subunits (5).

Two atoms of iron, presumably bound in heme prosthetic groups, are also components of myeloperoxidase (7). A brief report appeared (8) which indicated that the reduction and alkylation of canine myeloperoxidase under denaturing conditions resulted in the release of one of the heme prosthetic groups and the co-isolation of the remaining heme prosthetic group with the 57,500-M₂ subunit.

We report here that the reduction and alkylation of human myeloperoxidase under non-denaturing conditions results in the cleavage of myeloperoxidase in half. This new molecular species, which has been named hemi-myeloperoxidase, contains both subunit types, retains all heme prosthetic groups, and retains the full specific activity of the intact, native myeloperoxidase. According to the nomenclature of Monod et al. (9), hemi-myeloperoxidase constitutes the heavy-light-protomer (H-L-protomer) of native myeloperoxidase.

The availability of hemi-myeloperoxidase gave us the opportunity to examine the H-L-protomic structure of native myeloperoxidase. The axis of cleavage of native myeloperoxidase to form hemi-myeloperoxidase was determined using analytical ultracentrifugation techniques, and the number and sites of attachment of the disulfide bridge(s) which join the H-L-protomers was investigated. Some of these results have been reported briefly elsewhere (10).

MATERIALS AND METHODS

Myeloperoxidase Assay

Myeloperoxidase activity was assayed using a noncarcinogenic compound (11), tetramethylbenzidine. For the determination of specific activity, the reaction was carried out at room temperature in 50 mM sodium acetate buffer, pH 4.6, 0.88 mM TMB, and 5 mM H₂O₂. The reaction, initiated by the addition of enzyme, was followed by measuring the rate of appearance of the oxidation product of TMB at 655 nm with a Cary 14 spectrophotometer. One unit of activity is defined as the amount of enzyme necessary to decompose 1 μmol of H₂O₂/min.

Isolation Procedures

Isolation of White Blood Cells—Native myeloperoxidase was isolated from out-dated leukopheresis preparations, which provided on the order of 10⁶ white blood cells/batch. These out-dated preparations were obtained within 4 days of donation, during which time they were stored at 4 °C. Whole cells were obtained by sedimentation at 500 × g for 15 min. Contaminating red blood cells were removed from

* The abbreviations used are: H, heavy; L, light; TMB, tetramethylbenzidine; RZ, reinheit zahi; SDS, sodium dodecyl sulfate; CTAB, cetyltrimethylammonium bromide.
the cell pellets by the addition of distilled water for 2 min followed by the addition of 0.5 M NaCl to restore isotonicity (12). The whole cells were centrifuged for 15 min at 500 × g and the washing cycle was repeated until the pellet became off-white.

**Isolation of Native Myeloperoxidase**—The white blood cells were lysed by the addition of 0.5% CTAB (5) and cell debris was removed by centrifugation for 15 min at 15,000 × g at 5 °C. All subsequent centrifugations were carried out without a brake and for 30 min prior to centrifugation. The supernatant was treated with solid (NH₄)₂SO₄ to yield a final concentration of 50% saturation. This solution was held at 4 °C for 30 min and centrifuged to remove the precipitate. The resulting supernatant was treated with solid (NH₄)₂SO₄ to increase the concentration to 65% saturation, and was incubated at 4 °C for 30 min prior to centrifugation. This procedure, a modification of a method described by Agner (7), resulted in a pellet containing the bulk of the myeloperoxidase activity.

Myeloperoxidase precipitated by the addition of (NH₄)₂SO₄ was redissolved in a buffer containing 50 mM Tris, pH 7.0, and 0.5% CTAB (column buffer), and chromatographed on Sephadex G-150, superfine grade. To prepare this column, Sephadex G-150 was swollen at room temperature for 3 days in column buffer. The column, 2.5 × 30 cm, was washed with several volumes of column buffer before use and was run at room temperature.

**Sedimentation Velocity**—Native myeloperoxidase, in column buffer, was reduced by the addition of 0.4 M dithiothreitol to a final concentration of 40 mM, followed by incubation at room temperature for 1 h. A stock solution of 1 M iodoacetamide was added to a final concentration of 0.1 M and the solution was incubated at 4 °C for 1 h. A white precipitate formed and was removed by centrifugation at 15,000 × g for 5 min. The supernatant, containing the treated myeloperoxidase, was chromatographed on the Sephadex G-150 column.

**Gel Electrophoresis**

**Sodium Dodecyl Sulfate Gel Electrophoresis**—Protein solutions were analyzed by SDS-gradient pore gel electrophoresis using a 0.5% CTAB buffer. The stacking and running gel were used and the sample was applied directly to the gel. The running gel was composed of 50 mM sodium phosphate buffer, pH 7.0. The concentration of protein was determined by the absorbance at 280 nm in a Zeiss PMQ II spectrophotometer. All visible spectra were determined with a Cary 14 spectrophotometer. Absorbance at both wavelengths was monitored using a Zeiss MPQ spectrophotometer.

**Gel Staining**—SDS-gels and native gels were stained for protein by incubation at 50 °C for 4–5 h in a solution of 50% ethanol, 10% acetic acid, 0.5 g/liter of Coomassie Brilliant Blue R-250, and 0.5 g/liter of CuSO₄. The destaining was also done at 50 °C in 10% acetic acid, 10% ethanol.

Native gels were stained for peroxidase activity (16) by incubation at room temperature in a solution of 1 mM o-dianisidine for 5 min followed by incubation for 5 min in 10 mM H₂O₂.

**Analytical Ultracentrifugation**

**Sedimentation Velocity**—Sedimentation velocity experiments were performed using a Spinco model E ultracentrifuge, an AN-E rotor, and schlieren optics. All protein solutions were dialyzed against 50 mM Tris, pH 7.0, for 24 h at 4 °C. Final protein concentrations used ranged between 0.29 and 1.1 mg/ml. 50 mM Tris, pH 7.0, was used as a reference solution. All protein solutions were examined at 48,000 rpm. The sedimentation coefficients (s₂₀,₀) were calculated by the method of Schachman (17). Linear regression analysis was applied to the data in terms of the dependence of s₂₀,₀ on protein concentration. The intercept of this line at zero concentration defined the sedimentation coefficient, s₂₀,₀.

**Sedimentation Equilibrium**—Molecular weights were determined by the method of Yphantis (18). These experiments were done using a Spinco model E ultracentrifuge, an AN-D rotor, interference optics, and a 6-channel centerpiece. The protein solutions were the same samples used in the sedimentation velocity experiments and were used here at concentrations between 0.14 and 0.60 mg/ml. Native and hemi-myeloperoxidase were centrifuged at 15,000 and 20,000 rpm, respectively. All samples were centrifuged for at least 18 h. To ensure that equilibrium had been reached, photographs of the sedimented protein were taken after 18 h and again 2 h later. The molecular weights were determined as described by Chervenka (19).

**Determination of Axial Ratio**—The axial ratios of native and hemi-myeloperoxidase were determined from their experimentally determined sedimentation coefficients and molecular weights in 50 mM Tris, pH 7.0. The axial ratio of either protein was determined from the ratio of the frictional coefficient of that protein (f), calculated from experimentally determined parameters, and the frictional coefficient of a sphere with the same volume as that protein (f₀), a theoretical factor. f/f₀ was calculated as described by van Holde (20). A graph (20) related the ratio f/f₀ to the axial ratio of an unhydrated prolate ellipsoid of revolution.

**Characterization of Hemi-Myeloperoxidase**

**Extent of Alkylation**—The extent of alkylation was determined using [¹⁴C]iodoacetic acid with a specific activity of 5.1 mc/μmol as the alkylating agent during the isolation of hemi-myeloperoxidase. One hundred-μl aliquots of [³H]iodoacetic acid-treated myeloperoxidase were spotted onto 2.3 cm Whatman No. 3MM filters and dropped into a solution of 10% trichloroacetic acid at 4 °C. The filters were boiled 20 min in 5% trichloroacetic acid, washed with 85% ethanol for 20 min, rinsed briefly in acetone, and dried at room temperature. Each filter was put into a scintillation vial to which was added 50 μl of distilled water and 0.5 ml of Protosol. The vials were incubated at 37 °C for 18 h at which time 10 ml of Econofluor was added to each vial. The vials were cooled to 4 °C for 2 h and counted in a Beckman LS-4800 scintillation counter. The counting efficiency in Econofluor was 90.

**TABLE I**

<table>
<thead>
<tr>
<th>Preparation step</th>
<th>RZ</th>
<th>Activity Yield*</th>
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<tbody>
<tr>
<td>CTAB lysate</td>
<td>0.11</td>
<td>2.76</td>
</tr>
<tr>
<td>50% (NH₄)₂SO₄, supernatant</td>
<td>0.15</td>
<td>3.06</td>
</tr>
<tr>
<td>65% (NH₄)₂SO₄, supernatant</td>
<td>0.11</td>
<td>2.76</td>
</tr>
<tr>
<td>65% (NH₄)₂SO₄, pellet</td>
<td>0.26</td>
<td>15.4</td>
</tr>
<tr>
<td>Sephadex G-150, peak A</td>
<td>0.70</td>
<td>47.3</td>
</tr>
<tr>
<td>Peak A rechromatographed on Sephadex G-150</td>
<td>0.73</td>
<td>49.5</td>
</tr>
</tbody>
</table>

* Yield was determined as the per cent of peroxidase activity remaining at each step.
was 30%. Treatment of the filters was according to the method of Kluit (21).

Radioactivity in Gels—The radioactivity in polyacrylamide gels was determined by a modification of the method of Anderson and McClure (22). Approximately 20 μl of hemi-myeloperoxidase labeled with [3H]iodoacetic acid was subjected to SDS-gel electrophoresis. After staining with Coomassie Brilliant Blue R-250, the pertinent track was sliced into 59 0.5-mm pieces and each was put into a miniscintillation vial. In order to dissolve the gel matrix, each vial received 0.5 ml of 30% H2O2, was capped, and incubated at 37 °C for 24 h. This was followed by the addition of 5.0 ml of Aquasol 2 to each vial and further incubation at 4 °C for 18 h. The vials were then counted in a Beckman LS-55 scintillation counter. The counting efficiency of this system was 10%.

Materials

Tetramethylbenzidine was purchased from Polysciences, Inc., War-lington, PA. Hydrogen peroxide (30%) was purchased from Sigma. Iodoacetamide, cetyltrimethylammonium bromide, and o-dianisidine were purchased from Eastman. Sephadex were purchased from Pharmacia. Acrylamide and N,N'-methylenebisacrylamide, both of electrophoresis purity, and molecular weight standards were purchased from Bio-Rad. [3H]Iodoacetic acid (193.3 mCi/mmol), Protosol, Aquasol-2, and Econofluor were purchased from New Eng-

RESULTS

Isolation of Native Myeloperoxidase—White blood cells, isolated from leukophoresis preparations, were lysed with 0.5% CTAB to liberate myeloperoxidase. The enzyme was purified approximately 5–6-fold by (NH4)2SO4 precipitation (Table I). The partially purified myeloperoxidase was chromato-

graphed on a Sephadex G-150 column, and the elution profile obtained is shown in Fig. 1. The myeloperoxidase activity was limited to Peak A. This chromatographic procedure resulted in a 3-fold purification of the (NH4)2SO4-purified enzyme (Table I). Peak B had a spectrum which differed from that of myeloperoxidase, was devoid of enzymatic activity, and was not investigated further. Peak A fractions were pooled, concentrated by (NH4)2SO4 precipitation, and chromatographed a second time on Sephadex G-150. Under these conditions, a single symmetrical peak was obtained (data not shown). The myeloperoxidase thus obtained was concentrated by the addition of (NH4)2SO4, dialyzed against distilled H2O overnight and used in the following experiments as native myeloperoxidase.

Table I shows the degree of purification of myeloperoxidase at various stages of this isolation procedure. An accurate determination of the yield and purification factor of the cell lysis step was not possible due to the latency of myeloperoxidase activity and the inability to resuspend the pellet obtained after CTAB lysis for activity and protein assays. The purification factor of 17.9 was well within the range expected for myeloperoxidase, which has been estimated to compose 1–2% (23) or 5% (24) of the dry weight of neutrophils. The RZ of the purified myeloperoxidase was usually around 0.73 (0.71–0.74) and the specific activity was around 50 units/mg (44.6–56.5).

Native myeloperoxidase, with an RZ of 0.73, was analyzed by native gel electrophoresis. Two protein bands were evident when the gel was stained with Coomassie Brilliant Blue R-250, a major band with an RF of 0.24, and a minor band which remained on the top of the gel (Fig. 2). When the gel was stained for peroxidase activity, only one band appeared, corresponding to the protein band with an RF of 0.24. We conclude that the major band represents native myeloperoxidase. Isolated myeloperoxidase was determined to be 93% pure by scanning the gel stained for protein. This is in agreement with a value of 88% purity estimated by comparison of the RZ of this material to the RZ of crystalline canine myeloperoxidase, determined to be 0.82 (7).

Isolation of Hemi-Myeloperoxidase—Hemi-myeloperoxidase was obtained by reduction and alkylation of native myeloperoxidase under nondenaturing conditions and subsequent gel filtration on a Sephadex G-150 column. Fig. 3 shows the elution profile of this column. Peak I had the same elution volume and the same SDS-gel pattern (in the presence and absence of dithiothreitol) as native myeloperoxidase, and was therefore identified as unreduced, native myeloperoxidase. Peak II, for reasons to be presented below, has been desig-
Cleavage of Myeloperoxidase into Hemi-myeloperoxidase

The yield of hemi-myeloperoxidase ranged between 50–75% by this procedure. In addition, another component always appeared as a minor shoulder of Peak II. This component was devoid of both 430 nm absorbance and myeloperoxidase activity.

The Sephadex G-150 column used to isolate native and hemi-myeloperoxidases was calibrated with respect to molecular weight with conalbumin (86,180), prothrombin (74,000), horseradish peroxidase (44,050), lysozyme (17,200), and cytochrome c (12,400). Based on the elution volumes of these standards, the apparent molecular weights of native and hemi-myeloperoxidase were calculated to be 88,000 and 52,000, respectively. These figures do not reflect the true molecular weights of 153,000 and 78,000, as determined by analytical ultracentrifugation. Harrison et al. (5) have previously observed anomalous elution behavior of canine myeloperoxidase on Bio-Gel A-1.5M.

Table II compares the RZ values and specific activities of native myeloperoxidase and of the Peak I and Peak II proteins described in Fig. 3. As can be seen, hemi-myeloperoxidase retained the full specific activity of the native myeloperoxidase from which it was made. Comparable RZ values for native and hemi-myeloperoxidase indicate that there is no loss of heme prosthetic groups when native myeloperoxidase is converted to hemi-myeloperoxidase.

Spectra of Oxidized and Reduced Native and Hemi-Mye-
l Peroxidase — While maintenance of a high RZ value in hemi-
myeloperoxidase indicates that both hemes remained bound
in the half-enzyme preparation, visible spectra were generated
in order to compare the heme environment of native and
hemi-myeloperoxidase. The spectra of the oxidized and the
reduced forms of the enzymes were the same for native and
hemi-myeloperoxidase, as can be seen in Fig. 4. Thus it
appears, by the criterion of visible spectroscopy, that the
heme environments of hemi-myeloperoxidase and native
myeloperoxidase are identical.

Characterization of Native and Hemi-Myeloperoxidase on
Sodium Dodecyl Sulfate Polyacrylamide Gels — Fig. 5 shows
the analysis of native and hemi-myeloperoxidase on an SDS
gradient pore gel. Tracks 3 and 6 contained molecular weight
standards which were used to calculate the apparent molec-
ular weights of the isolated proteins. Track 1 of Fig. 4 con-
tained native myeloperoxidase without added dithiothreitol.
The major protein bands corresponded to apparent molecular
weights of 120,000 and 105,000 as determined by a plot of the
dependence of log molecular weight on the distance of migra-
tion through the gel of the standards. Track 2 contained
native myeloperoxidase with added dithiothreitol. The major
cbands corresponded to M, 57,500 and 14,000. Track 4 con-
tained hemi-myeloperoxidase without added dithiothreitol.
This material had major protein bands at M, = 57,000 and
55,500. Track 5 contained hemi-myeloperoxidase with added
dithiothreitol. This material had major protein bands at M, =
57,500 and 14,000.

A comparison of Tracks 1 and 4, native and hemi-myelo-
peroxidase, respectively, without added dithiothreitol, shows
that hemi-myeloperoxidase is much smaller than native my-
eloperoxidase. The apparent molecular weights of the major
protein bands were 120,000 and 105,000 for native myelo-
peroxidase and 67,000 and 55,500 for hemi-myeloperoxidase:
myeloperoxidase migrated with about half of the appar-
ent molecular weight of native myeloperoxidase. Tracks 2 and
5, native and hemi-myeloperoxidase, respectively, with added
dithiothreitol, demonstrate that the hemi-myeloperoxidase

FIG. 7 (left). Dependence of sedimentation coefficient of na-
tive myeloperoxidase on concentration. The sedimentation coeffi-
cients of various concentrations of native myeloperoxidase (MPO)
determined by centrifugation of native myeloperoxidase at
48,000 rpm in a model E ultracentrifuge. The enzyme was suspended
in 50 mM Tris, pH 7.0. The data were analyzed by linear regression.
The sedimentation coefficient of native myeloperoxidase extrapolated
to zero concentration was 8.0 S.

Fig. 8 (center). Dependence of sedimentation coefficient of
hemi-myeloperoxidase on concentration. The sedimentation coeffi-
cients of various concentrations of hemi-myeloperoxidase were
determined by centrifugation of hemi-myeloperoxidase at 48,000 rpm
in a model E ultracentrifuge. The enzyme was suspended in 50 mM
Tris, pH 7.0. The data were analyzed by linear regression. The
sedimentation coefficient of hemi-myeloperoxidase (Hemi-MPO)
extrapolated to zero concentration was 4.3 S.

Sedimentation Velocity — Both native and hemi-myelo-
peroxidase appeared homogeneous in these studies (Fig. 6).
The sedimentation coefficient, s20,w, was determined for native
myeloperoxidase at concentrations between 0.29 and 0.96 mg/ml.
The dependence of s20,w on concentration for native myelo-
peroxidase is shown in Fig. 7. Extrapolation of these data to
zero concentration by linear regression analysis gave a sedi-
mentation coefficient, s20,w, of 8.0 S for native myeloperoxidase.
This is in agreement with the figure of 7.9 S for canine
myeloperoxidase as determined by Ehrenberg and Agner (25).
The s20,w was determined for hemi-myeloperoxidase at con-
centrations between 0.34 and 1.1 mg/ml. The relationship of
s20,w and concentration of hemi-myeloperoxidase is shown in
Fig. 8. The s20,w for hemi-myeloperoxidase was found to be 4.3 S.

Sedimentation Equilibrium — Sedimentation equilibrium
experiments were carried out for native myeloperoxidase at
0.14, 0.3, and 0.5 mg/ml and for hemi-myeloperoxidase at 0.15,
0.26, and 0.56 mg/ml. The partial specific volume (v) of human
myeloperoxidase was assumed to be the same as canine mye-
loperoxidase, which was determined by Ehrenberg and Agner
(25) to be 0.731. In addition, v was assumed to be the same for
both native and hemi-myeloperoxidase. The molecular weight
of native myeloperoxidase was determined to be 153,000 ±4,000
and the molecular weight of hemi-myeloperoxidase was
determined to be 78,000 ±2,000. Both molecular weights were
independent of protein concentration.

Axial Ratio Determination — To determine the axis of
cleavage of native myeloperoxidase upon reduction to form
hemi-myeloperoxidase, the axial ratio of each protein was
determined. The sedimentation coefficients and molecular
weights determined by sedimentation velocity and sedimenta-
ion equilibrium, respectively, were used in this determi-

radioactivity profile of [3H]iodoacetic acid-labeled
e~i-m},6-myeloperoxidase analyzed by SDS-gel electropho-
resis. Approximately 20 l~g of [3H]iodoacetic acid-labeled
hemi-myeloperoxidase, treated with 40 mM dithiothreitol, was subjected to
SDS-gradient pore gel electrophoresis. The gel was stained with
Coomassie Brilliant Blue R-250. The pertinent track was excised from
the gel and sliced into 59, 1.5-mm pieces. The gel slices were treated
as described under "Materials and Methods." Data are presented as
counts per min above background (10 cpm); the counting efficiency
in this system was 10%.
tion. The ratios, $f/f_0$, were calculated to be 1.28 and 1.54 for native and hemi-myeloperoxidase, respectively (Table III). The axial ratio of native myeloperoxidase was found to be 5.1 while that of hemi-myeloperoxidase was 10.1, indicating that native myeloperoxidase was cleaved along its long axis in the formation of hemi-myeloperoxidase.

**Extent and Sites of Alkylation**—To determine the extent of alkylation which took place in the process of forming hemi-myeloperoxidase, [\textsuperscript{3}H]iodoacetic acid was used in the reduction and alkylation procedure. The [\textsuperscript{3}H]carboxymethylated hemi-myeloperoxidase was isolated by Sephadex G-150 chromatography. Using the molecular weights of 153,000 for native myeloperoxidase and 78,000 for hemi-myeloperoxidase, the figures determined by sedimentation equilibrium, the number of carboxymethylated groups formed per mol of protein was obtained. As seen in Table IV, Peak I material, or unreduced, native myeloperoxidase, incorporated 0.4 mol of carboxymethyl group/mol of native myeloperoxidase. Peak II material, which represents hemi-myeloperoxidase, incorporated 2.4 mol of carboxymethyl groups/mol of native myeloperoxidase or 1.2 mol of carboxymethyl groups/mol of hemi-myeloperoxidase. These data are interpreted as indicating that a single disulfide bond was broken in the native enzyme to produce the heme-enzyme.

To determine the alkylation sites and thus the subunits involved in the disulfide bridge connecting the two half-enzymes, the column fractions of hemi-myeloperoxidase which had been alkylated with [\textsuperscript{3}H]iodoacetic acid were pooled and precipitated with solid ammonium sulfate to a final concentration of 65% saturation. The precipitate was removed by centrifugation and resuspended in and dialyzed against distilled water. The dialyzed hemi-myeloperoxidase was subjected to SDS-gradient pore gel electrophoresis in the presence of diethiothreitol. After staining, the pattern typical for hemi-myeloperoxidase plus diethiothreitol was observed which consisted of peptide bands corresponding to both the heavy ($M_r = 57,500$) and light ($M_r = 14,000$) subunits. The gel was sliced and the gel slices were counted as described under “Materials and Methods.” The radioactivity profile of this gel is shown in Fig. 9. As can be seen, 90% of the radioactivity was present in the 57,500-$M_r$ subunit of myeloperoxidase. It therefore appears that in native myeloperoxidase, a single disulfide bridge links the two large subunits, and this disulfide is reduced and alkylated in the process of converting native myeloperoxidase to hemi-myeloperoxidase.

A further examination of this conclusion was undertaken. It was reasoned that, if a single disulfide bond is broken to form hemi-myeloperoxidase, then reduced but unalkylated hemi-myeloperoxidase, then  reduced but unalkylated hemi-myeloperoxidase to hemi-myeloperoxidase.

**Table III**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Native myeloperoxidase</th>
<th>Hemi-myeloperoxidase</th>
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</thead>
<tbody>
<tr>
<td>Molecular weight*</td>
<td>153,000 ± 4,000</td>
<td>78,000 ± 2,000</td>
</tr>
<tr>
<td>Sedimentation coefficient</td>
<td>8.0</td>
<td>4.3</td>
</tr>
<tr>
<td>$f/f_0$</td>
<td>1.28</td>
<td>1.54</td>
</tr>
<tr>
<td>Axial ratio</td>
<td>5.1</td>
<td>10.1</td>
</tr>
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</table>

* ± S.D.

**Table IV**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>[\textsuperscript{3}H]carboxymethyl groups/153,000 $M_r$</th>
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<tbody>
<tr>
<td>Peak I (myeloperoxidase)</td>
<td>0.4</td>
</tr>
<tr>
<td>Peak II (hemi-myeloperoxidase)</td>
<td>2.4</td>
</tr>
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</table>

**Discussion**

A procedure for the isolation of native myeloperoxidase from normal human neutrophils has been described. This procedure utilized detergent extraction, differential ammonium sulfate precipitation, and gel exclusion chromatography for the relatively quick isolation, with good yield, of human myeloperoxidase. Myeloperoxidase was isolated by this method to 89–93% purity, as judged both by RZ values (Table I) and native gel electrophoresis (Fig. 2). The purified enzyme myeloperoxidase might be able to dimerize, but should not be able to form a higher multimer of hemi-myeloperoxidase. To test this hypothesis, native myeloperoxidase was reduced in the presence of 80 mM diethiothreitol and hemi-myeloperoxidase was isolated by chromatography on Sephadex G-150 in the presence of 1 mM diethiothreitol. Oxygen was bubbled through the hemi-myeloperoxidase solution for 22 h at room temperature. The protein solution was alkylated with iodoacetamide and chromatographed on Sephadex G-150. The elution profile of this column is shown in Fig. 10. In addition to hemi-myeloperoxidase, a re-formed species with the same elution volume as native myeloperoxidase was observed. This re-formed species had the same specific activity as both the original native enzyme and hemi-myeloperoxidase. No higher multimer of hemi-myeloperoxidase was produced by this procedure. This corroborates the evidence obtained using [\textsuperscript{3}H]iodoacetic acid, that a single disulfide bridge is involved in joining the two halves of human myeloperoxidase. A schematic representation of our present view of native myeloperoxidase is depicted in Fig. 11.
Consistently had a specific activity of approximately 50 units/ml.

The cationic detergent, CTAB, has been used in the isolation of myeloperoxidase from guinea pig (26), canine (5), and human neutrophils (16, 27). In all those instances, myeloperoxidase was released from whole cells by the action of this detergent. We found CTAB to be useful also for the chromatography of human myeloperoxidase on Sephadex G-150. Myeloperoxidase was found to bind to the column matrix when chromatography was conducted at neutral pH, whereas the addition of 0.5% CTAB to the column buffer prevented this binding. The affinity of myeloperoxidase for the column matrix may have been due solely to charge effects, as chromatography of human myeloperoxidase on Sephadex G-150 equilibrated at pH 4.5 did not require the addition of CTAB (data not shown).

Purified native myeloperoxidase was reduced with dithiothreitol, alkylated with iodoacetamide, and chromatographed on Sephadex G-150. Two principal products resulted which retained both the high RZ value and the full specific activity of native myeloperoxidase (Fig. 3). Peak I material was identified as unreduced, native myeloperoxidase. Peak II material was examined further with respect to its size, subunit composition, and heme environment. Based on the results discussed below, Peak II is a half-molecule of native myeloperoxidase, referred to as hemi-myeloperoxidase.

Although both native myeloperoxidase and hemi-myeloperoxidase eluted as sharp, symmetrical peaks when chromatographed on Sephadex G-150 (Fig. 3), their elution volumes reflected apparent molecular weights much smaller than the molecular weights determined by sedimentation equilibrium. Native myeloperoxidase and hemi-myeloperoxidase eluted with apparent molecular weights of 88,000 and 56,000, respectively, whereas the molecular weights determined by sedimentation equilibrium were 153,000 and 78,000, respectively. Anomalous behavior of canine myeloperoxidase on gel exclusion chromatography has been reported previously (5).

Native myeloperoxidase was subjected to SDS-gel electrophoresis in the absence of a reducing agent and displayed major protein bands corresponding to molecular weights of 120,000 and 105,000. When the Peak II (hemi-myeloperoxidase) material was electrophoresed under the same conditions (Fig. 5), major protein bands appeared at Mr = 67,000 and 55,500; approximately half of that found with native myeloperoxidase. Electrophoresis of either native myeloperoxidase or the Peak II material on SDS-gels in the presence of dithiothreitol gave two major protein bands at Mr = 57,000 and 14,000. The ratio of the staining intensities of these two bands were 14:1 for native myeloperoxidase and 13:1 for the Peak II material.

Peak II material was designated hemi-myeloperoxidase, the half-enzyme form of myeloperoxidase, containing one 57,500 Mr, subunit, one 14,000 Mr subunit, and a full complement of heme prosthetic groups for the following reasons: 1) The Peak II material eluted from the Sephadex G-150 column as a sharp, symmetric peak, indicating the presence of a single species (Fig. 3). 2) Analysis of the molecular size of the Peak II material by either gel filtration, SDS-gel electrophoresis, or analytical ultracentrifugation showed it to be approximately one-half the size of native myeloperoxidase (Figs. 3 and 5, Table III). 3) SDS-gel electrophoresis of the Peak II material demonstrated that both the 57,000 Mr, and the 14,000 Mr, subunit types were present (Fig. 5). 4) The ratio of these subunit types in the Peak II material was the same as that found in native myeloperoxidase. 5) The RZ of the Peak II material was the same as that of native myeloperoxidase, indicating that no heme was released from the enzyme during the procedure of reduction and alkylation (Table II).

Because reduction and alkylation of myeloperoxidase under denaturing conditions resulted in the release of half of the heme groups (3), the procedure to form hemi-myeloperoxidase might alter the heme environment. The spectra of oxidized and reduced hemi-myeloperoxidase were examined to determine the nature of the heme environment. As can be seen in Fig. 4, native myeloperoxidase and hemi-myeloperoxidase had identical spectra in either the oxidized or reduced state. This indicates that no alteration of the heme environment occurred as a result of the procedure of reduction and alkylation.

Of particular interest is the fact that hemi-myeloperoxidase retained the full specific activity of native myeloperoxidase. This characteristic of hemi-myeloperoxidase strongly suggests that the two halves of the native enzyme can act as independent catalytic components during the assay for peroxidase activity. This is not to suggest that the two halves are equivalent catalytic components. Some data have been presented which suggest that differences between the two halves exist in either heme structure, heme linkage to the protein, or protein environment at the site of heme attachment (7, 8, 26, 28). We therefore conclude that whatever heme reactivity differences exist between the two halves of native myeloperoxidase, these differences are not due to an interaction between the two halves.

Experiments aimed at the determination of the axis of cleavage of native myeloperoxidase during reduction and alkylation necessarily characterized the molecular weight, sedimentation coefficient, and axial ratio of both native and hemi-myeloperoxidase. The molecular weight of hemi-myeloperoxidase, determined by sedimentation equilibrium, was 78,000 ± 2,000. This confirms the half-enzyme nature of hemi-myeloperoxidase, as the molecular weight of native myeloperoxidase was determined to be 153,000 ± 4,000. The sedimentation coefficient of native myeloperoxidase was found to be 8.0 S and had a dependence upon protein concentration indicative of an elongated molecule (20). The sedimentation coefficient of hemi-myeloperoxidase was found to be 4.3 S and had a dependence upon protein concentration indicative of a rapidly associating-dissociating system (20). This suggests that there are sites of noncovalent recognition lying at the interface of the H-L-promoters in addition to the disulfide bridge which covalently joins them. The axial ratio of native myeloperoxidase was calculated to be 5:1 while that of hemi-myeloperoxidase was 10:1, having assumed an unhydrated prolate ellipsoid of revolution. Assuming an unhydrated oblate ellipsoid, the axial ratios become 6:1 and 12:1, respectively. The molecular weight, sedimentation coefficient, and axial ratio of native human myeloperoxidase, reported here, are in complete agreement with the same determinants of native canine myeloperoxidase (25).

Investigation of the axial ratios of native myeloperoxidase and hemi-myeloperoxidase and of the number and sites of the disulfide bridges which join the two H-L-promoters have helped clarify the quaternary structure of myeloperoxidase. The axial ratios of native myeloperoxidase and hemi-myeloperoxidase of 5:1 and 10:1, respectively, strongly suggest that the two H-L-promoters lie side by side along their long axes to form the native enzyme. By using [3H]iodoacetic acid as the alkyllating reagent in the preparation of hemi-myeloperoxidase we were able to show that a single disulfide bridge joins the two halves of the native enzyme (Table IV). This conclusion was corroborated by the finding that reduced, unalkylated hemi-myeloperoxidase re-forms no higher multimer than native myeloperoxidase when exposed to oxygen (Fig. 10). Gel electrophoresis of [3H]iodoacetic acid-labeled hemi-myeloperoxidase showed that the reducible disulfide bond...
links the two heavy subunits of native myeloperoxidase (Fig. 9). This is the first evidence of a close spatial orientation of the heavy subunits of myeloperoxidase.

Dimethylsuberimidate treatment has been reported to cross-link the light subunits, but not the heavy subunits of canine myeloperoxidase (6). There are two possible interpretations of these data. First, the quaternary structures of canine and human myeloperoxidase may be different. These two enzymes have been shown to have the same molecular weight, sedimentation coefficient, axial ratio, and iron content, as well as similar subunit molecular weights (both have heavy subunits of 57,500 M₉ with a light subunit of 14,000 M₉ for human myeloperoxidase and 10,500 M₉ for canine myeloperoxidase (5). Although it is possible, it seems unlikely that myeloperoxidase from these two sources would have almost identical compositions and yet have radically different quaternary structures. A second interpretation of the cross-linking data is that the heavy-subunit:heavy-subunit interface does not happen to have suitable amino acid residues (primarily lysines) in the proper orientation to accommodate cross-linking by dimethylsuberimidate.

If the quaternary structures of canine and human myeloperoxidase are the same, then the linear H-L-L-H structure proposed earlier (5) is not adequate to describe the new evidence that the heavy subunits are joined by a disulfide bridge. With the information presently available, it is impossible to estimate the proportion of the H-L-promoter:H-L-promoter interface which is contributed by the L-subunit:L-subunit interface. Therefore, the model we have proposed (Fig. 11) expresses only the hypothesis that there are L-subunit:L-subunit and H-subunit:H-subunit interfaces and should not be interpreted as a statement of the extent of these shared surfaces.

Investigation of the quaternary structure of myeloperoxidase is progressing steadily and several significant observations have been made. The enzyme is made up of four subunits, two heavy and two light. Myeloperoxidase contains two heme prosthetic groups, and at least one of the heme groups and all of the carbohydrate is bound to the heavy subunit of myeloperoxidase (5). The two light subunits share an interface (5) and the two heavy subunits share an interface at least large enough to accommodate a disulfide bridge. And lastly, the two H-L-protomers lie side by side along their long axes to form native myeloperoxidase.

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