

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

(Received for publication, September 10, 1980, and in revised form, November 14, 1980)

Patricia C. Andrews† and Norman I. Krinsky

From the Department of Biochemistry and Pharmacology, Tufts University School of Medicine, Boston, Massachusetts 02111

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

Hemi-myeloperoxidase retains both heme groups and contains both subunit types ($M_r = 57,500$ 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]iodoacetic 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 nondenaturing conditions results in the formation of hemi-myeloperoxidase, a catalytically active heavy-light protomer of native myeloperoxidase.

Myeloperoxidase (donor: H_2O_2 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 H_2O_2 and a halide such as chloride, iodide, or bromide (3, 4).

Canine myeloperoxidase contains four subunits; two heavy ($M_r = 57,500$) and two light ($M_r = 10,500$) polypeptide chains (5). Human myeloperoxidase has been reported to have sub-

unit molecular weights of 54,000 and 14,000 (6). Dimethylsulberimidate 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- M_r subunit.

We report here that the reduction and alkylation of human myeloperoxidase under nondenaturing 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-protomeric¹ 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 4.5, 0.88 mM TMB, and 5 mM H_2O_2 . 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 H_2O_2 /min.

Isolation Procedures

Isolation of White Blood Cells—Native myeloperoxidase was isolated from out-dated leukaphoresis preparations, which provided on the order of 10^{10} 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 $500 \times g$ for 15 min. Contaminating red blood cells were removed from

* This work was supported in part by Grant PCM 77-1026 from the National Science Foundation and Grant AM-17745 from National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Present address, Division of Hematology, Department of Medicine, Tufts-New England Medical Center Hospitals, 171 Harrison Ave., Boston, MA 02111.

¹ The abbreviations used are: H, heavy; L, light; TMB, tetramethylbenzidine; RZ, reinheit zahl; SDS, sodium dodecyl sulfate; CTAB, cetyltrimethylammonium bromide.

the cell pellets by the addition of distilled water for 2 min followed by the addition of 0.6 M NaCl to restore isotonicity (12). The whole cells were centrifuged for 15 min at $500 \times g$ 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 $15,000 \times g$ at 5°C . All subsequent centrifugations were carried out under these conditions. The supernatant was treated with solid $(\text{NH}_4)_2\text{SO}_4$ 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 $(\text{NH}_4)_2\text{SO}_4$ 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 $(\text{NH}_4)_2\text{SO}_4$ 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.

Isolation of Hemi-Myeloperoxidase—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 $8,000 \times g$ for 2 min in a Brinkmann 3200 Microfuge. The supernatant, containing the treated myeloperoxidase, was chromatographed on the Sephadex G-150 column.

Spectrophotometry

All visible spectra were determined with a Cary 14 spectrophotometer scanning from 700 to 360 nm. The spectrum of the oxidized enzyme was obtained using a 0.5 mg/ml preparation of untreated enzyme in 50 mM sodium phosphate buffer, pH 7.0. To obtain reduced enzyme, several grains of sodium hydrosulfite were added to the solution (13).

The concentration of protein was determined by the absorbance at 280 nm using a Zeiss PMQ II spectrophotometer. A standard curve was generated with cytochrome c, which gave an absorbance at 280 nm of 1.56 for a 1 mg/ml solution.

RZ is defined as the ratio of absorbance at 430 and 280 nm (A_{430}/A_{280}). The RZ is used as a parameter of purity of myeloperoxidase (7). Absorbance at both wavelengths was monitored using a Zeiss MPQ II spectrophotometer.

Gel Electrophoresis

Sodium Dodecyl Sulfate Gel Electrophoresis—Protein solutions were analyzed by SDS-gradient pore gel electrophoresis using a modification of the method of O'Farrell (14). The sample buffer contained 1% SDS, 0.06 M Tris, pH 6.8, and 4% glycerol. Bromophenol blue was used as the tracking dye. All samples, including those treated with 40 mM dithiothreitol, were incubated in sample buffer for 15 min at room temperature before they were applied to the gel. Anomalous banding patterns were observed when unreduced myeloperoxidase was boiled prior to electrophoresis. Molecular weight standards were consistently reduced with 40 mM dithiothreitol prior to loading onto the gel. The running gel consisted of an 8–22% acrylamide gradient with 0.375 M Tris, pH 8.0, and 0.2% SDS. The running buffer (pH unadjusted) was 0.05 M Tris, 0.38 M glycine, and 0.1% SDS. Electrophoresis was carried out at 170 V until 30 min after the tracking dye ran off the gel, the entire process taking 3 h. Gels were incubated overnight at 50°C in a solution containing 10% acetic acid in 50% ethanol to remove the SDS in preparation for staining.

Native Gel Electrophoresis—Native gel electrophoresis was carried out using the method of Reisfeld *et al.* (15) except that no stacking gel was used and the sample was applied directly to the running gel. The running gel was 6% acrylamide and cast in tubes. These gels were run at pH 4.2 with the electrodes arranged such that protein migrated through the gel toward the cathode. The gels were prerun at 6 mA/gel for $1\frac{1}{2}$ h. The samples were applied and the gels were run at 6 mA/gel until the tracking dye, pyronin G, reached the bottom of the tube, which usually took about $1\frac{1}{2}$ h. R_F was determined as the ratio of the distance of protein migration and the distance of tracking dye migration.

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 CuSO_4 . 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 H_2O_2 .

Gel Scanning—Tube gels and 9 mm vertical slices of slab gels which had previously been stained with Coomassie Brilliant Blue R-250 were scanned at 620 nm using an ISCO model 1310 gel scanner.

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 ($s_{20,w}$) were calculated by the method of Schachman (17). Linear regression analysis was applied to the data in terms of the dependence of $s_{20,w}$ on protein concentration. The intercept of this line at zero concentration defined the sedimentation coefficient, $s_{20,w}^0$.

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 insure 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 calculated 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 (f_0), a theoretical factor. f/f_0 was calculated as described by van Holde (20). A graph (20) related the ratio f/f_0 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 [^3H]iodoacetic acid with a specific activity of 5.1 mCi/mmol 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 95% 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-350 scintillation counter. The counting efficiency in Econofluor

TABLE I
Purification of native myeloperoxidase

Preparation step	RZ	Activ- ity	Purifi- cation factor	Yield ^a
		units/mg		%
CTAB lysate	0.11	2.76	1.0	100
50% $(\text{NH}_4)_2\text{SO}_4$ supernatant	0.15	3.06	1.1	86
65% $(\text{NH}_4)_2\text{SO}_4$ supernatant	0.11	0.76		17
65% $(\text{NH}_4)_2\text{SO}_4$ pellet	0.26	15.4	5.6	72
Sephadex G-150 Peak A	0.70	47.3	17.1	61
Peak A rechromatographed on Sephadex G-150	0.73	49.5	17.9	56

^a 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 Palmitor (21).

Radioactivity in Gels—The radioactivity in polyacrylamide gels was determined by a modification of the method of Anderson and McClure (22). Approximately 20 μ l of hemi-myeloperoxidase labeled with [3 H]iodoacetic acid was subjected to SDS-gel electrophoresis. After staining with Coomassie Brilliant Blue R-250, the pertinent track was sliced into 59 1.5-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% H_2O_2 , 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-350 scintillation counter. The counting efficiency of this system was 10%.

Materials

Tetramethylbenzidine was purchased from Polysciences, Inc., Warrington, PA. Hydrogen peroxide (30%) was purchased from Sigma. Iodoacetamide, cetyltrimethylammonium bromide, and *o*-dianisidine were purchased from Eastman. Sephadex G-150, superfine grade, was purchased from Pharmacia. Acrylamide and *N,N'*-methylenebisacrylamide, both of electrophoresis purity, and molecular weight standards were purchased from Bio-Rad. [3 H]iodoacetic acid (193.3 mCi/mmol), Protosol, Aquasol-2, and Econofluor were purchased from New England Nuclear. Bovine prothrombin was provided through the courtesy of Dr. Barbara Furie (New England Medical Center Hospital).

Outdated white blood cells were provided by both the New England Medical Center Hospital Blood Bank and the Sidney Farber Blood Component Laboratory, Boston, MA.

RESULTS

Isolation of Native Myeloperoxidase—White blood cells, isolated from leukapheresis preparations, were lysed with 0.5% CTAB to liberate myeloperoxidase. The enzyme was purified approximately 5–6-fold by $(\text{NH}_4)_2\text{SO}_4$ precipitation (Table I). The partially purified myeloperoxidase was chromatographed 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 $(\text{NH}_4)_2\text{SO}_4$ -precipitated enzyme (Table I). Peak B had a spectrum which differed from that of myeloperoxidase, was devoided of enzymatic activity, and was not investigated further. Peak A fractions were pooled, concentrated by $(\text{NH}_4)_2\text{SO}_4$ 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 $(\text{NH}_4)_2\text{SO}_4$, dialyzed against distilled H_2O 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 R_F 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 R_F 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 89% 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-

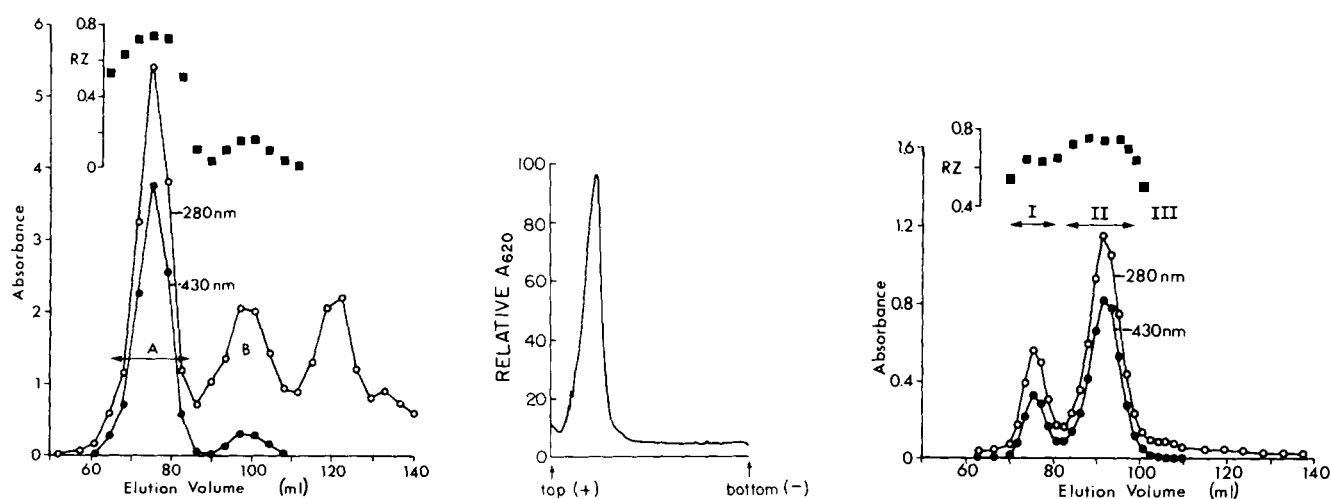


FIG. 1 (left). Elution profile of first Sephadex G-150 column. The absorbance at 280 (○) and 430 (●) nm was determined for fractions (0.9 ml) collected from Sephadex G-150 chromatography of crude myeloperoxidase. The RZ (A_{430}/A_{280}) (■) was also determined for each fraction. Peak A contained the myeloperoxidase activity. Fractions with an RZ > 0.4 (arrows) were pooled for further purification. The void volume of this column was 58 ml.

FIG. 2 (center). Scan of native gel containing native myeloperoxidase. Twenty μ g of native myeloperoxidase were electropho-

resed on native gels. Gels were stained with Coomassie Brilliant Blue R-250 and scanned at 620 nm.

FIG. 3 (right). Elution profile of hemi-myeloperoxidase on Sephadex G-150. Myeloperoxidase which had been treated with dithiothreitol and iodoacetamide was chromatographed on Sephadex G-150. The absorbance at 280 (○) and 430 (●) nm and the RZ (■) of each fraction (0.9 ml) was determined. Arrows show which fractions were pooled for further analysis. The void volume of this column was 58 ml.

nated as the half enzyme form of myeloperoxidase, or 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

TABLE II
Purification of hemi-myeloperoxidase on Sephadex G-150

Fraction	RZ	Units/ mg	M_r^a
Native myeloperoxidase	0.73	49.5	88,000
Peak I (myeloperoxidase)	0.64	41.3	88,000
Peak II (hemi-myeloperoxidase)	0.74	51.2	52,000

^a Determined from elution volume on Sephadex G-150.

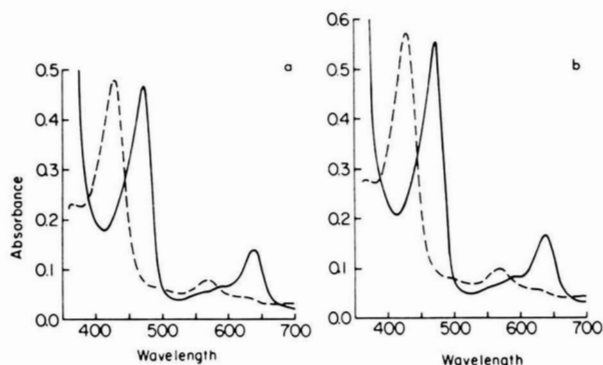


FIG. 4. Spectra of oxidized and reduced native and hemi-myeloperoxidase. The spectra of native (a) and hemi-myeloperoxidase (b) were determined at 0.44 and 0.50 mg/ml, respectively. Each enzyme was suspended in 50 mM sodium phosphate, pH 7.0, and the oxidized spectrum (dashed lines) was monitored. For the reduced spectrum (solid lines) of each enzyme, several grains of sodium hydrosulfite were added.

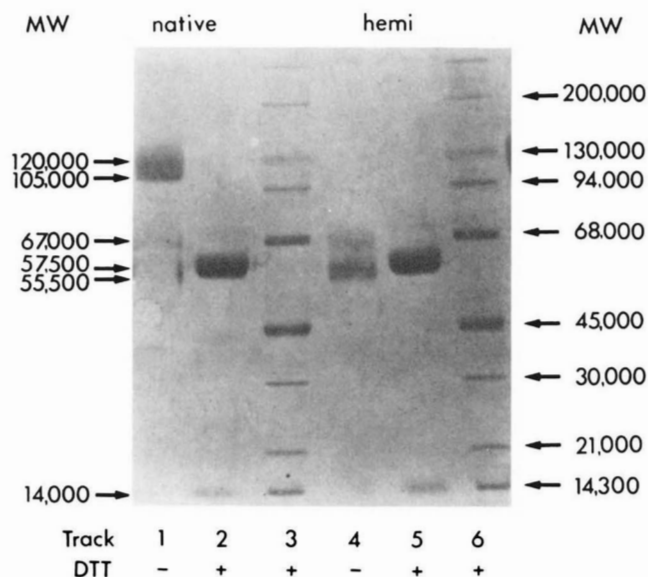


FIG. 5. SDS-gradient pore gel analysis of native and hemi-myeloperoxidase. Twenty-three μ g of native myeloperoxidase (tracks 1 and 2) and 25 μ g of hemi-myeloperoxidase (tracks 4 and 5) were applied to the gel. Tracks 2 and 5 represent enzyme which had been reduced with 40 mM dithiothreitol (DTT) prior to being loaded onto the gel. Tracks 3 and 6 contain molecular weight standards; myosin (200,000), β -galactosidase (130,000), phosphorylase b (94,000), bovine serum albumin (68,000), ovalbumin (45,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (21,000), and lysozyme (14,300). Approximately 1 μ g of each molecular weight standard was used per track and the standards were reduced with 40 mM dithiothreitol before being applied to the gel.

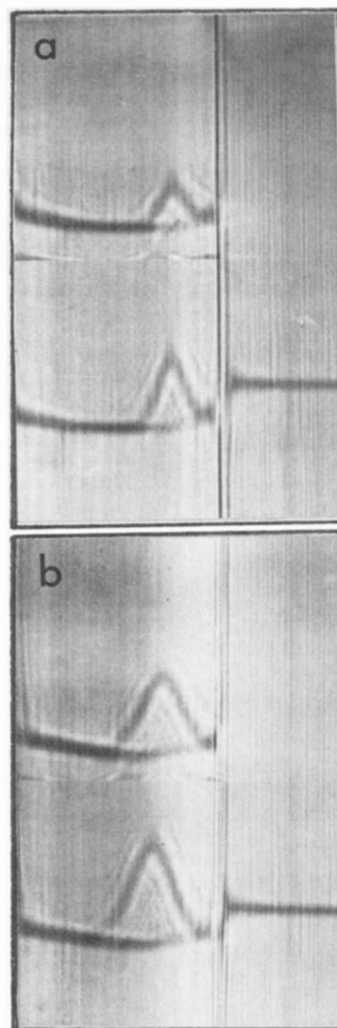


FIG. 6. Native and hemi-myeloperoxidase in sedimentation velocity experiments. Using schlieren optics, photographs were taken of native and hemi-myeloperoxidase during centrifugation at 48,000 rpm. The direction of sedimentation was from right to left. (a) native myeloperoxidase at 0.29 mg/ml (top) or 0.53 mg/ml (bottom), in 50 mM Tris, pH 7.0. (b) hemi-myeloperoxidase at 1.11 mg/ml (top) or 0.89 mg/ml (bottom) in 50 mM Tris, pH 7.0.

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-

loperoxidase—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 molecular weights of the isolated proteins. Track 1 of Fig. 4 contained 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 migration through the gel of the standards. Track 2 contained native myeloperoxidase with added dithiothreitol. The major bands corresponded to M_r 57,500 and 14,000. Track 4 contained hemi-myeloperoxidase without added dithiothreitol. This material had major protein bands at M_r = 67,000 and 55,500. Track 5 contained hemi-myeloperoxidase with added dithiothreitol. This material had major protein bands at M_r = 57,500 and 14,000.

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

preparation contained both the 57,500- and the 14,000- M_r subunit types found in native myeloperoxidase. In addition, the ratio of the staining intensities at 620 nm of the 57,500- and 14,000- M_r protein bands was found to be 14:1 for native myeloperoxidase and 13:1 for hemi-myeloperoxidase.

Sedimentation Velocity—Both native and hemi-myeloperoxidase appeared homogeneous in these studies (Fig. 6). The sedimentation coefficient, $s_{20,w}$, was determined for native myeloperoxidase at concentrations between 0.29 and 0.96 mg/ml. The dependence of $s_{20,w}$ on concentration for native myeloperoxidase is shown in Fig. 7. Extrapolation of these data to zero concentration by linear regression analysis gave a sedimentation coefficient, $s_{20,w}^0$ 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 $s_{20,w}$ was determined for hemi-myeloperoxidase at concentrations between 0.34 and 1.1 mg/ml. The relationship of $s_{20,w}$ and concentration of hemi-myeloperoxidase is shown in Fig. 8. The $s_{20,w}^0$ 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 0.6 mg/ml and for hemi-myeloperoxidase at 0.15, 0.26, and 0.56 mg/ml. The partial specific volume (\bar{v}) of human myeloperoxidase was assumed to be the same as canine myeloperoxidase, which was determined by Ehrenberg and Agner (25) to be 0.731. In addition, \bar{v} was assumed to be the same for both native and hemi-myeloperoxidase. The molecular weight of native myeloperoxidase was determined to be $153,000 \pm 4,000$ and the molecular weight of hemi-myeloperoxidase was determined to be $78,000 \pm 2,000$. 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 sedimentation equilibrium, respectively, were used in this determina-

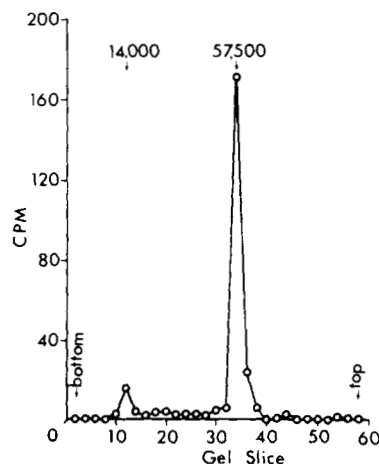
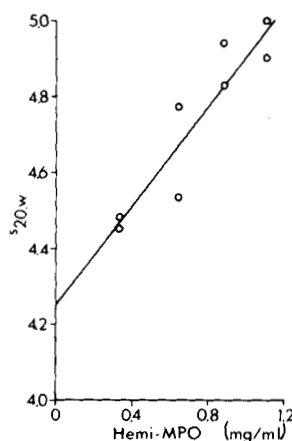
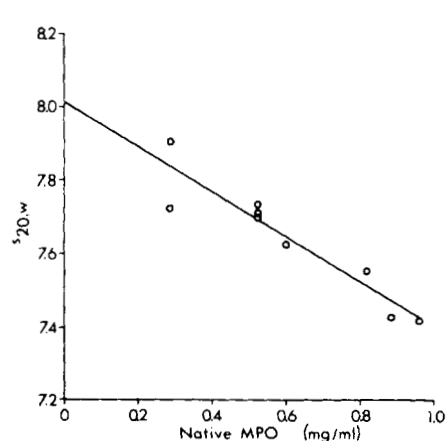


FIG. 7 (left). Dependence of sedimentation coefficient of native myeloperoxidase on concentration. The sedimentation coefficients of various concentrations of native myeloperoxidase (MPO) were determined by centrifugation of native myeloperoxidase at 48,000 rpm in a model E ultracentrifuge. The enzyme 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 coefficients 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.

FIG. 9 (right). Radioactivity profile of [^3H]iodoacetic acid-labeled hemi-myeloperoxidase analyzed by SDS-gel electrophoresis. Approximately 20 μg 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, [^3H]iodoacetic acid was used in the reduction and alkylation procedure. The [^3H]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 hemi-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 [^3H]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 dithiothreitol. After staining, the pattern typical for hemi-myeloperoxidase plus dithiothreitol was obtained 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-

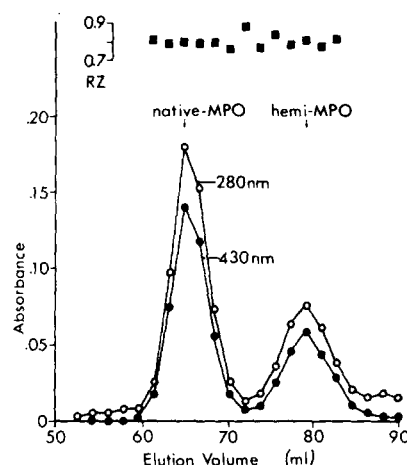


FIG. 10. Elution profile of oxidized hemi-myeloperoxidase on a Sephadex G-150 column. Unalkylated hemi-myeloperoxidase which had been oxygenated for 22 h was alkylated, concentrated, and chromatographed on Sephadex G-150. The absorbance at 280 (○) and 430 (●) nm and the RZ (■) of the fractions (0.9 ml) were determined. The elution volumes of native and hemi-myeloperoxidase were determined by chromatography of these species.

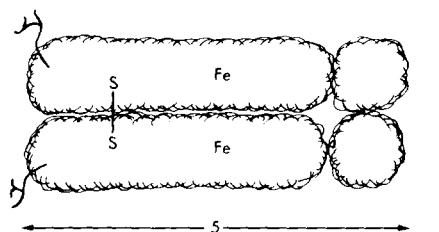


FIG. 11. Model of structure of native myeloperoxidase. This structure of native myeloperoxidase is proposed to accommodate the experimental data which has been reported concerning the structure and composition of native myeloperoxidase.

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 dithiothreitol and hemi-myeloperoxidase was isolated by chromatography on Sephadex G-150 in the presence of 1 mM dithiothreitol. 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 [^3H]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.

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

TABLE III
Physical parameters of native and hemi-myeloperoxidase determined by analytical ultracentrifugation

Parameter	Native myeloperoxidase	Hemi-myeloperoxidase
Molecular weight ^a	153,000 ± 4,000	78,000 ± 2,000
Sedimentation coefficient	8.0	4.3
f/f_0	1.28	1.54
Axial ratio	5:1	10:1

^a ± S.D.

TABLE IV
[^3H]Iodoacetic acid bound to isolated fractions of hemi-myeloperoxidase preparation

Fraction	[^3H]carboxymethyl groups/153,000 M_r
Peak I (myeloperoxidase)	0.4
Peak II (hemi-myeloperoxidase)	2.4

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 major 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, while 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 $M_r = 67,000$ 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 $M_r = 57,500$ 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- M_r subunit, one 14,000- M_r 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 size 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 57,500- M_r and the 14,000- M_r 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 (5), 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 $78,000 \pm 2,000$. This confirms the half-enzyme nature of hemi-myeloperoxidase, as the molecular weight of native myeloperoxidase was determined to be $153,000 \pm 4,000$. 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 alkylating 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.

Dimethylsuberimide 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 M_r with a light subunit of 14,000 M_r for human myeloperoxidase and 10,500 M_r 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 dimethylsuberimide.

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.

Acknowledgments—We gratefully acknowledge receipt of a partially purified sample of myeloperoxidase kindly supplied by Dr. Inge

Olsson, University Hospital of Lund, Lund, Sweden. In addition, we would like to express our appreciation to Dr. Richard Siegel and Dr. Robert Wolfert for their assistance in the analytical ultracentrifugation experiments.

REFERENCES

1. Klebanoff, S. J., and Clark, R. A. (1978) *The Neutrophil: Function and Clinical Disorders*, North-Holland, Amsterdam
2. Klebanoff, S. J. (1975) *Semin. Hematol.* **12**, 117-142
3. Klebanoff, S. J. (1967) *J. Exp. Med.* **126**, 1063-1078
4. Klebanoff, S. J. (1968) *J. Bacteriol.* **95**, 2131-2138
5. Harrison, J. E., Pabalan, S., and Schultz, J. (1977) *Biochim. Biophys. Acta* **493**, 247-259
6. Olsson, I., Olofsson, T., and Odeberg, H. (1972) *Scand. J. Haematol.* **9**, 483-491
7. Agner, K. (1958) *Acta Chem. Scand.* **12**, 89-94
8. Harrison, J. E., and Schultz, J. (1978) *Fed. Proc.* **37**, 1514
9. Monod, J., Wyman, J., and Changeux, J.-P. (1965) *J. Mol. Biol.* **12**, 88-118
10. Andrews, P. C., and Krinsky, N. I. (1979) *Fed. Proc.* **38**, 461
11. Holland, V. R., Saunders, B. C., Rose, F. L., and Walpole, A. L. (1974) *Tetrahedron* **30**, 3299-3302
12. Levine, P. H., Weinger, R. S., Simon, J., Scoon, K. L., and Krinsky, N. I. (1976) *J. Clin. Invest.* **57**, 955-963
13. Zgliczyński, J. M., Stelmaszyńska, T., Ostrowski, W., Naskalski, J., and Sznajd, J. (1968) *Eur. J. Biochem.* **4**, 540-547
14. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007-4021
15. Reisfeld, R. A., Lewis, U. J., and Williams, D. E. (1962) *Nature* **195**, 281-283
16. Bakkenist, A. R. J., Wever, R., Vulsma, T., Plat, H., and Van Gelder, B. F. (1978) *Biochim. Biophys. Acta* **524**, 45-54
17. Schachman, H. K. (1959) *Ultracentrifugation in Biochemistry*, Academic Press, London
18. Yphantis, D. A. (1964) *Biochemistry* **3**, 297-317
19. Chervenka, C. H. (1970) *A Manual of Methods*, Spinco Division, Beckman Instruments, Palo Alto, Calif.
20. Van Holde, K. E. (1971) *Physical Biochemistry*, pp. 80-81, 98-108, Prentice-Hall, Inc., Englewood Cliffs, N. J.
21. Palmiter, R. D. (1973) *J. Biol. Chem.* **248**, 2095-2106
22. Anderson, L. E., and McClure, W. O. (1973) *Anal. Biochem.* **51**, 173-179
23. Agner, K. (1941) *Acta Physiol. Scand.* **II**, Suppl. 8, 1-62
24. Schultz, J., and Kaminker, K. (1962) *Arch. Biochem. Biophys.* **96**, 465-467
25. Ehrenberg, A., and Agner, K. (1958) *Acta Chem. Scand.* **12**, 95-100
26. Odajima, T., and Yamazaki, I. (1972) *Biochim. Biophys. Acta* **284**, 360-367
27. Merrill, D. P. (1980) *Prep. Biochem.* **10**, 133-150
28. Schultz, J., and Rosenthal, S. (1972) in *Molecular Evolution* (Rohlfing, D. L., and Oparin, A. I., eds) pp. 271-289, Plenum Publishing Co., N. Y.