Isolation and Properties of Human Neutrophil Myeloperoxidase†

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ABSTRACT: Human leukocyte myeloperoxidase has been purified to homogeneity by a three-step procedure which includes dialysis of a granule extract against low-salt buffer, Sephadex G-75 chromatography, and carboxymethylcellulose chromatography. The final product was homogeneous when examined by acid polyacrylamide gel electrophoresis and sedimentation equilibrium ultracentrifugation. The molecular weight determined by the latter procedure was 118 000. With or without reduction of the protein by 2-mercaptoethanol, subunits were formed which migrated as a single band after sodium dodecyl sulfate gel electrophoresis. With reduction, the molecular weight of the apparently identical subunits was 59 000, and 42 000 without reduction. Other general properties of human leukocyte myeloperoxidase, including amino acid composition, amino terminal sequence analysis, and absorption spectra, are also reported. Myeloperoxidase, in the presence of hydrogen peroxide and chloride ion, and no other substrate, autoinactivates. After completion of the inactivation reaction, several oxidizable amino acids in the enzyme are modified, and the absorption peak at 430 nm disappears. The presence of a substrate of the myeloperoxidase system (α-1-proteinase inhibitor), or of high concentration of chloride ion, completely protects the enzyme from autoinactivation.

During acute inflammation, polymorphonuclear leukocytes collect in large numbers by directed migration from the vascular space into the tissues. Within these cells are cytoplasmic granules containing oxidative and hydrolytic enzymes. Although the primary role of these enzymes is the destruction of microorganisms and debris within the phagocyte vacuole, it has become increasingly clear they also participate in extracellular reactions.

Activation of the polymorphonuclear leukocyte by several stimuli leads to secretion or leakage of granule enzymes into the extracellular space (Zurier et al., 1973; Klebanoff & Clark, 1978; Henson et al., 1978). Among the major granule enzymes released are elastase and cathepsin G (Starkey, 1977), each of which is capable of degrading lung tissue (Reilly & Travis, 1980). This destruction is usually controlled by proteinase inhibitors which can diffuse from the plasma into the tissues.

A third granule enzyme released is myeloperoxidase (Henson, 1971). The burst of oxidative metabolism which accompanies stimulation of leukocytes releases various activated oxygen species, including H_2O_2 (Root et al., 1975; Levine et al., 1976). Thus, the microbicidal system of myeloperoxidase, H_2O_2, and halides is simultaneously present, extracellularly (Klebanoff, 1975), and this has been shown to exert effects on many other cells (Edelson & Cohn, 1973; Clark et al., 1975; Clark & Klebanoff, 1977, 1979a,b).

In the accompanying paper, we describe in detail the effect of myeloperoxidase on a plasma proteinase inhibitor, α-1-proteinase inhibitor (α-1-PI),[1] a preliminary report of which has already been published (Matheson et al, 1979). The effect of myeloperoxidase has also been demonstrated recently in another laboratory (Carp & Janoff, 1980). In order to perform the current studies, it was necessary to purify the enzyme to homogeneity. Myeloperoxidase has already been isolated by many procedures and from various sources (Agner, 1958; Newton et al., 1965; Zgliczynski et al., 1968; Olsson et al., 1972; Desser et al., 1972). However, the reported techniques were either long and tedious, the enzyme was only partially purified, or the recovery was not satisfactory.

In the current paper, we report a purification procedure which involves only three steps. The enzyme is purified to homogeneity, as shown by acid and $NaDodSO_4$ gel electrophoresis, as well as by analytical ultracentrifugation, with excellent recovery. The properties of the enzyme, which differ in some respects from other reports, are also examined, including some of the parameters involved in autoinactivation by oxidation.

Experimental Procedures

Materials

Human polymorphonuclear leukocytes were obtained from normal subjects by leukapheresis of healthy donors or from patients with polycythemia vera. α-1-PI was prepared by procedures developed in this laboratory (Pannell et al, 1974). Porcine pancreatic elastase was from Sigma Chemical Co.

Carboxymethylcellulose-52 was obtained from Whatman Chemicals and Sephadex G-75 (superfine) from Pharmacia Fine Chemicals Inc. Hydrogen peroxide (30%), 4-aminoantipyrine, and cyanogen bromide were purchased from J. T. Baker Chemical Co. Dithioerythritol and dithiothreitol were from Sigma Chemical Co. All other chemicals were of analytical grade.

Methods

Leukocytes and leukocyte granules were isolated either from whole blood or from preparations obtained by leukapheresis (Baugh & Travis, 1976). Granule extracts were prepared by homogenization of granule pellets in 0.2 M sodium acetate buffer and 1.0 M NaCl, pH 4.0. The granule extract was centrifuged at 30 000*g* for 10 min and the pellet rehomogenized until the presence of enzyme could no longer be detected by assay. Three extractions were usually found to be adequate.

Purification of Human Leukocyte Myeloperoxidase. Leukocyte granule extracts were dialyzed at 4 °C overnight against 0.05 M Tris-HCl, 0.1 M NaCl, and 0.001 M dithiothreitol, pH 8.0. The precipitate, containing most of the

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[1] Abbreviations used: α-1-PI, α-1-proteinase inhibitor; $NaDodSO_4$, sodium dodecyl sulfate; CM-cellulose, carboxymethylcellulose.

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granule cathepsin G and elastase, was removed by centrifugation at 1100*g* for 10 min. The green supernatant, which contained all of the myeloperoxidase, was concentrated to approximately 15 mL and then applied to a column of Sephadex G-75 (superfine) equilibrated with 0.05 M Tris-HCl, 0.1 M NaCl, and 0.001 M dithiothreitol, pH 8.0. At this stage, two major proteins of molecular weights 81 000 and 59 000 were present in the first peak eluted, as judged by $NaDodSO_4$ gel electrophoresis. All of the myeloperoxidase activity was found in this peak.

In the final step, the fractions containing myeloperoxidase activity were pooled and dialyzed against 0.02 M sodium acetate and 0.1 M NaCl, pH 5.0, overnight at 4 °C. The sample was then applied to a column of CM-cellulose equilibrated with the same buffer and washed until the A_{280} was less than 0.1. The myeloperoxidase was isolated by a linear salt gradient from 0.1 to 0.4 M NaCl, the activity being eluted at 0.3 M NaCl. The enzyme was stored frozen and was found to be stable for several months, even with repeated freezing and thawing.

Enzyme Assay. Myeloperoxidase activity was measured spectrophotometrically by using 4-aminoantipyrine as the hydrogen donor. Solutions of 4-aminoantipyrine (0.5 mg/mL in 0.17 M phenol) were stable for several weeks in the dark at room temperature. Solutions of hydrogen peroxide (1.7 mM) were prepared fresh daily in the desired buffer. Myeloperoxidase activity was followed by addition of a suitably diluted enzyme preparation (0.02–0.1 mL) to 0.5 mL of hydrogen peroxide and 0.40–0.48 m of sodium phosphate buffer, pH 6.1. Activity was recorded as an increase in A_{510} per minute. One unit was defined as a change in absorbance of 1.0 optical density units per minute at 510 nm.

Protein concentration was determined by the Lowry procedure (Lowry et al., 1951) with bovine serum albumin as standard, and by A_{280} in later stages of the purification.

Assay for Myeloperoxidase Inactivation. In order to follow the autocatalytic inactivation of myeloperoxidase, aliquots of the enzyme were incubated at 22 °C in a reaction mixture containing 0.2 M sodium phosphate buffer, 0.16 M NaCl, and 0.37 mM H_2O_2, pH 6.1. In some experiments, the NaCl concentration was increased to 0.64 or 1.28 M, or α-1-PI was added. Aliquots were removed at 1-min intervals and added to a catalase solution (0.32 μM), which was sufficient to stop the myeloperoxidase reaction but did not interfere in the subsequent antipyrine assay.

Gel Electrophoresis. Acid polyacrylamide gel electrophoresis was carried out according to the procedure of Brewer & Ashworth (1969). Electrophoresis was performed at pH 4.3 in a 7.5% gel and stained with 1% Coomassie Blue G.

$NaDodSO_4$–polyacrylamide gel electrophoresis was performed as described in ORTEC Life Science Note 13. Electrophoresis utilized a 0.1 M Tris-glycine buffer, pH 8.3, and 1% $NaDodSO_4$, ± 2-mercaptoethanol. A 9% gel was employed.

Amino Acid Analysis. Samples for amino acid analysis (65–250 μg) were hydrolyzed in vacuo in constant-boiling HCl for 24, 48, and 72 h at 105 °C. Amino acid analysis was carried out with a Beckman Model 119CL amino acid analyzer. Half-cystine and methionine were determined after performic acid oxidation (Hirs, 1967). Methionine sulfoxide content was determined after cyanogen bromide cleavage of the oxidized or control protein, followed by acid hydrolysis in the presence of dithioerythritol (Shechter et al., 1975). Tryptophan was measured by the method of Oliveira et al. (1979).

Table I: Purification of Human Leukocyte Myeloperoxidase

fractionation step	total protein (mg)[a]	total activity (unit)[b]	recovery (%)	sp act. (units/mg)	purification	A_{426}/A_{280}
crude extract	166	9520	100	57.3	1.0	0.08
crude extract after dialysis	98	9410	99	96.0	1.6	0.18
Sephadex G-75 column	35	8568	90	244.8	4.2	0.32
CM-cellulose column	15	7512	79	501.0	8.9	0.65

[a] Based on absorbance at 280 nm. [b] Based on enzymatic activity by using 4-aminoantipyrine as hydrogen donor. 1 unit = A_{510} of 1.0 OD/min.

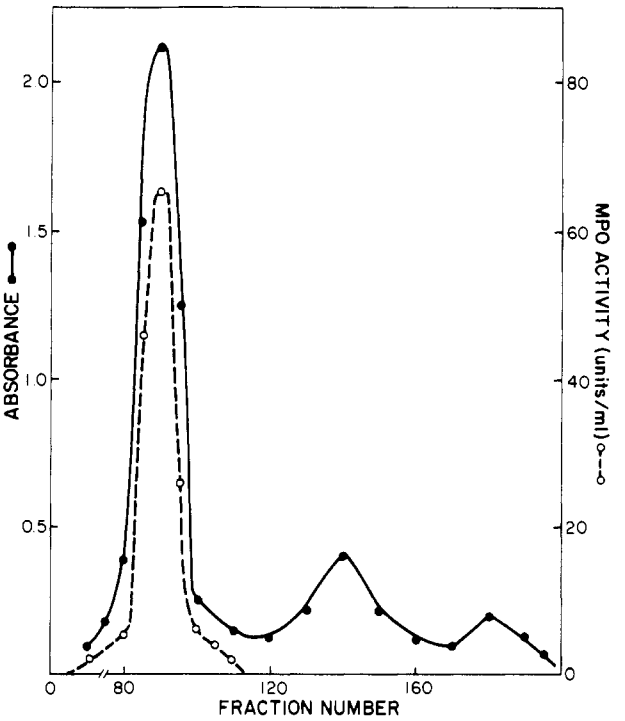

FIGURE 1: Chromatography of human leukocyte granule extracts on Sephadex G-75. The column (5.0 × 90 cm) was equilibrated with 0.5 M Tris-HCl, 0.1 M NaCl, and 0.001 M dithiothreitol, pH 8.0. After sample application (15 mL of dialyzed, concentrated granule extract), the flow was adjusted to 18 mL/h. Each fraction was dialyzed against 0.05 M Tris-HCl and 0.1 M NaCl, pH 8.0, before assay.

Ultracentrifuge Analyses. Sedimentation equilibrium experiments were performed at an initial protein concentration of 0.25 mg/mL in 0.1 M sodium phosphate buffer, pH 7.0 (Yphantis, 1964).

Results

Preparation of Human Leukocyte Myeloperoxidase. The recovery of human leukocyte myeloperoxidase activity at each stage of purification is given in Table I. Dialysis against buffer containing a relatively low salt concentration (0.1 M NaCl) served to remove most of the elastase and cathepsin G, which generally require 0.15 M NaCl or higher to remain in solution (Baugh & Travis, 1976).

The green supernatant remaining after dialysis and centrifugation separated into one major and several minor components after Sephadex G-75 chromatography (Figure 1), with all of the enzyme activity appearing in the void volume of the column. Other proteins, including lysozyme and traces of elastase, were retarded and thus removed from the myeloperoxidase fraction.

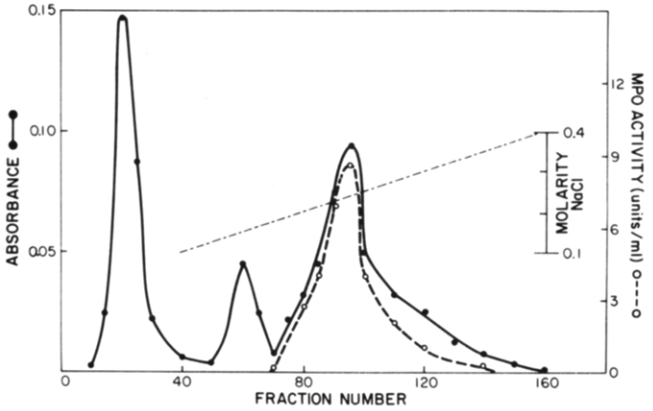

FIGURE 2: CM-celluose chromatography of human leukocyte myeloperoxidase fractions obtained from Sephadex G-75 chromatography. The column (1.9 × 15 cm) was equilibrated with 0.02 M sodium acetate and 0.1 M NaCl, pH 5.0, and eluted with a linear gradient in the same buffer from 0.1 to 0.4 M NaCl. Total gradient was 500 mL.

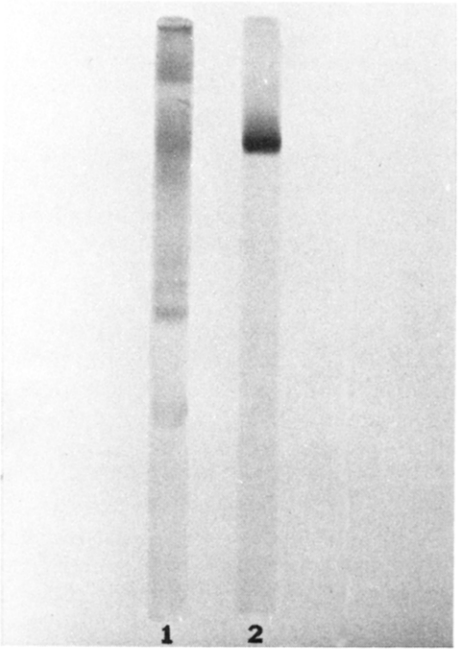

FIGURE 3: Acid gel electrophoresis of human granule extract fraction and purified human myeloperoxidase. Proteins were stained with 1% Coomassie Brilliant Blue G-250. Elecrophoresis was at pH 4.3 in a standard 7.5% acrylamide gel. Direction of migration is from anode (top) to cathode (bottom). (1) Dialyzed crude extract (200 μg); (2) myeloperoxidase fraction from CM-cellulose chromatography (50 μg).

When the pooled void volume fractions were chromatographed on CM-cellulose at pH 5.0, using a linear salt gradient for elution, essentially homogeneous myeloperoxidase was obtained (Figure 2). The final yield of activity was excellent and represented 79% of the original activity in the leukocyte homogenate.

Ultracentrifuge Studies on Human Myeloperoxidase. Sedimentation equilibrium experiments indicated that the protein was homogeneous and had a molecular weight of 118 000 when a partial specific volume of 0.738, calculated from the amino acid composition, was used.

An extinction coefficient ($E^{1\%}_{280}$) was computed from ultracentrifuge experiments by using interference optics (Babul & Stellwagen, 1969) and found to be 14.5.

Polyacrylamide Gel Electrophoresis of Myeloperoxidase. Acid gel electrophoresis revealed a single band after CM-cellulose chromatography, as shown in Figure 3. $NaDodSO_4$ gel electrophoresis of reduced, denatured samples showed two bands after Sephadex G-75 chromatography (Figure 4A, lane 2) and one band after the CM-cellulose step (Figure 4A, lane 3). The apparent molecular weight of myeloperoxidase by using standards for comparison was found to be 59 000.

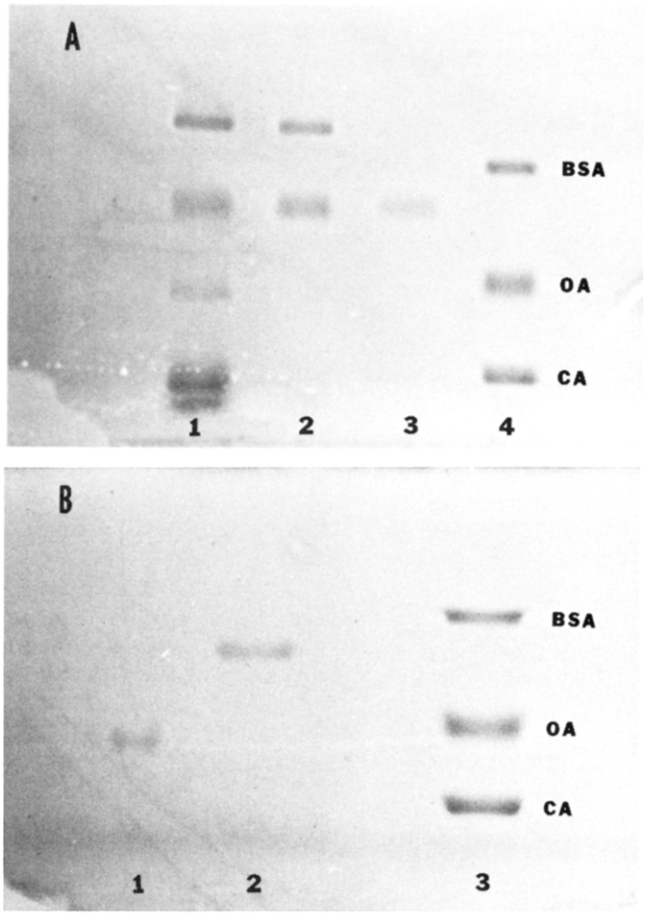

FIGURE 4: (A) $NaDodSO_4$ gel electrophoresis of human leukocyte granule extract and various fractions at each stage of purification of myeloperoxidase. (1) Dialyzed crude extract (50 μg); (2) active fractions from Sephadex G-75 chromatography; (3) active fractions from CM-cellulose chromatography; (4) standard proteins: bovine serum albumin (BSA) 68 000; ovalbumin (OA) 43 000; carbonic anhydrase (CA) 30 000. A 9% polyacrylamide gel was utilized. (B) $NaDodSO_4$ gel electrophoresis of purified leukocyte myeloperoxidase under reduced and nonreduced conditions. (1) Enzyme without 2-mercaptoethanol added (25 μg); (2) enzyme with 2-mercaptoethanol added (25 μg); (3) standard proteins, same as in (A).

In the absence of reducing agents, however, the apparent molecular weight of myeloperoxidase was only 42 000 (compare Figure 4B, lanes 1 and 2). Since sedimentation equilibrium experiments indicated a molecular weight of 118 000 for the enzyme, we assume that myeloperoxidase exists as two identical subunits of 59 000 molecular weight. We assume that without the presence of reducing agents the subunits were more compact and appeared to be smaller than their true molecular weight would indicate.

Amino Acid Composition. The amino acid analysis of myeloperoxidase is given in Table II. The levels of half-cystine are rather high and may account for the apparent compactness of the subunits in the absence of 2-mercaptoethanol. The composition, in general, agrees with those reported elsewhere (Odajima & Yamazaki, 1972; Harrison et al., 1977; Bakkenist et al., 1978).

Absorption Spectra of Human Leukocyte Myeloperoxidase. The absorption spectra of oxidized and reduced myeloperoxidase are given in Figure 5. The oxidized enzyme was

Table II: Primary Composition of Human Leukocyte Myeloperoxidase

amino acid	residues per mol			
	a	*b*	*c*	*d*
lysine	24	33	24	39
histidine	12	13	14	14
arginine	96	112	122	107
aspartic acid	132	116	152	136
threonine	56	42	64	58
serine	54	42	74	61
glutamic acid	136	80	108	129
proline	84	73	100	
glycine	76	65	68	80
alanine	72	63	70	75
half-cystine	24	26	20	
valine	48	60	48	71
methionine	24	18	22	31
isoleucine	32	30	48	48
leucine	60	110	128	121
tyrosine	24	19	34	27
phenylalanine	48	48	56	55
tryptophan	12		34	

[a] This paper (residues per 118 000 mol wt). [b] Odajima & Yamazaki (1972) (residues per 139 000 mol wt); pig. [c] Harrison et al. (1977) (residues per 136 000 mol wt); dog. [d] Bakkenist et al. (1978) (residues per 140 000 mol wt); human.

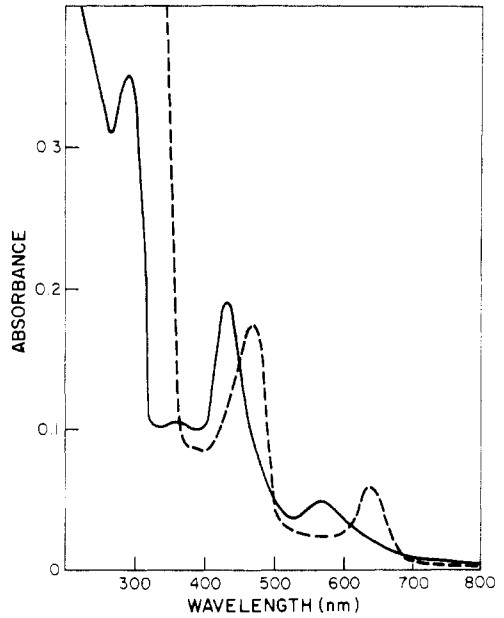

FIGURE 5: Absorption spectra of oxidized and reduced human leukocyte myeloperoxidase. The spectra were made with the enzyme in 0.1 M sodium phosphate buffer, pH 7.0. Oxidized, native enzyme (—); enzyme after addition of sodium dithionite (---).

characterized by an absorption maximum at 426 nm and minor peaks at 570 and 359 nm. The reduced enzyme showed maxima at 470 and 637 nm. These spectra are, essentially, in agreement with those reported elsewhere (Zgliczynski et al., 1968; Bakkenist et al., 1978). The absorption ratio (A_{430}/A_{280}) has usually been accepted as a sign of purity and is shown at each stage of purification in Table I.

Amino-Terminal Sequence of Human Leukocyte Myeloperoxidase. In order to determine whether myeloperoxidase was composed of identical or nonidentical subunits, 5 mg of enzyme was subjected to amino terminal sequence analysis. A single protein sequence was found for the first eight residues as follows: Val-Asp-Pro-Glu-Thr-Met-Arg-Val. This suggests, but does not prove, that the two subunits are identical.

Oxidative Inactivation of Myeloperoxidase. Human leukocyte myeloperoxidase is recognized for its ability to kill

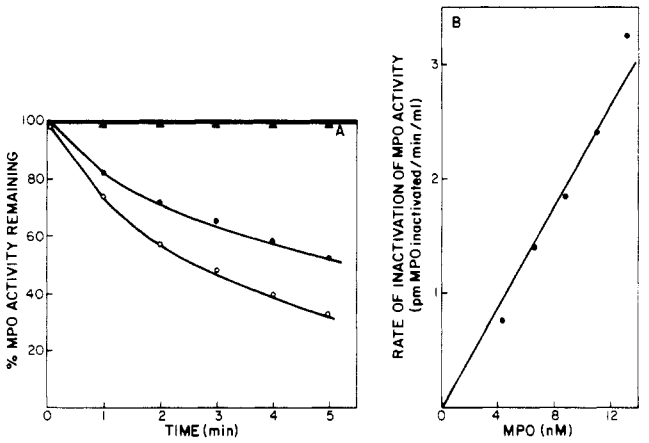

FIGURE 6: Dependence of the autoinactivation of myeloperoxidase on time and myeloperoxidase concentration. Myeloperoxidase was incubated at room temperature with 0.37 mM hydrogen peroxide, 0.16 M NaCl, and 0.2 M sodium phosphate buffer, pH 6.1. At 1-min intervals, an aliquot was removed and added to a solution of catalase (final concentration 0.32 μM) to stop the reaction. Each aliquot was assayed as described under Methods. (A) Percent loss of myeloperoxidase activity with time: (●) 4.21 nM myeloperoxidase; (○) 12.63 nM myeloperoxidase; (▲) 12.63 nM myeloperoxidase, NaCl omitted. (B) Dependence of rate of inactivation of myeloperoxidase on enzyme concentration.

bacteria in the presence of hydrogen peroxide and halide ion (Klebanoff, 1975). In the accompanying paper (Matheson et al., 1980), we describe in detail the conditions under which the major plasma proteinase inhibitor, α-1-PI, is inactivated by myeloperoxidase, peroxide, and halide ion. Inactivation, in this case at least, was caused by oxidation of two methionyl residues, one of which was at the reactive site of the inhibitor. When α-1-PI was omitted, however, myeloperoxidase activity was lost. The rate of activity loss proceeded with time and depended on myeloperoxidase concentration, as shown in Figure 6A, In the absence of chloride ion, no myeloperoxidase activity was lost. These results indicate that inactivation was caused by products of the myeloperoxidase reaction. Figure 7 illustrates the complete protection of myeloperoxidase from self-destruction by the presence of α-1-PI, at 0.16 M NaCl. The same protection was also given by higher concentrations of NaCl, 0.64 or 1.28 M, alone. The inactivation of myeloperoxidase by the product of its action on taurine and protection from it by addition of increasing concentrations of chloride ion have been observed by others (Naskalski, 1977).

When the concentration of myeloperoxidase was intense enough, a green color could be easily detected, and during the incubation with hydrogen peroxide and chloride ion, it gradually disappeared. Comparison of the spectra of native and inactivated myeloperoxidase (Figure 8) indicated that the Soret band had entirely disappeared. The heme groups are tightly bound in the native enzyme (Harrison & Schultz, 1978), but these results indicated that, nevertheless, they were totally removed from the enzyme molecule during the inactivation.

Loss of the heme groups alone could render myeloperoxidase inactive, but since they are normally tightly bound some damage to the protein molecule would be expected. We were able to demonstrate that oxidation of two methionyl residues had occurred when α-1-PI was inactivated (Matheson et al., 1980). Thus, amino acid analyses were performed on native and inactive myeloperoxidase in order to determine if similar events had occurred here. Table III presents the results of these analyses. All of the methionine, cysteine, and tryptophan residues, as well as some of the tyrosyl residues, have been destroyed. Also, half of the histidyl residues are missing. No

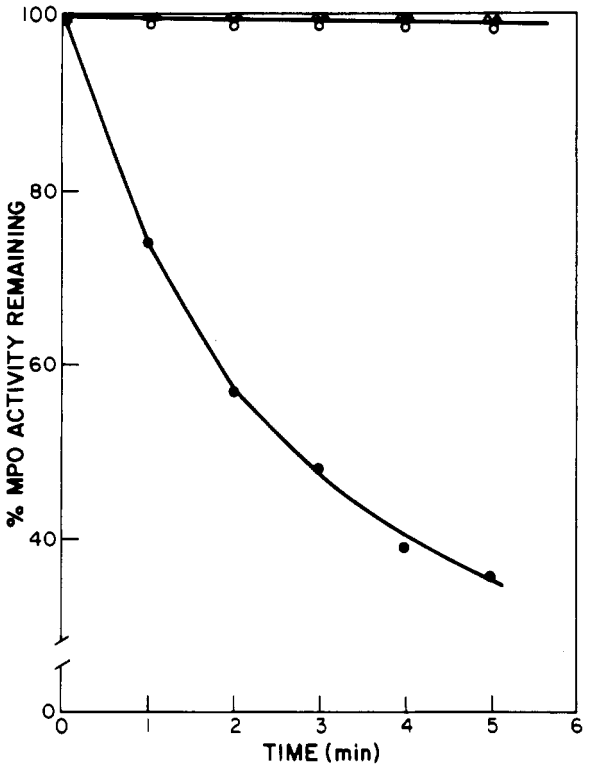

FIGURE 7: Protection of myeloperoxidase by α-1-PI and by high concentrations of NaCl. Myeloperoxidase (12.63 nM) was incubated at room temperature with 0.37 nM hydrogen peroxide, 0.16 M NaCl, and 0.2 M sodium phosphate, pH 6.1. Where indicated, α-1-PI (2.68 μM) was added to the standard reaction mixture, or the NaCl concentration was increased to 0.64 or 1.28 M. The reaction was stopped with catalase, and assays were performed as described under Methods. (●) 0.16 M NaCl; (○) 1.28 M NaCl; (▲) 0.64 M NaCl; (△) 2.68 μM α-1-PI.

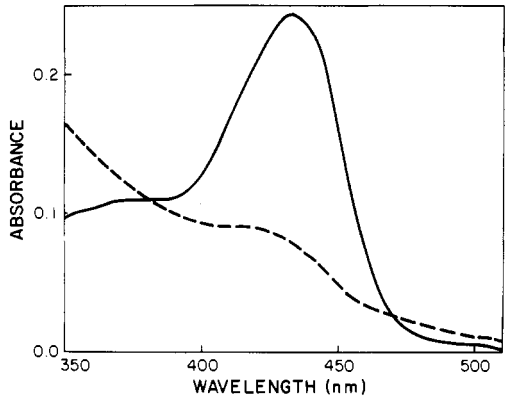

FIGURE 8: Absorption spectra of native and inactivated myeloperoxidase. Native myeloperoxidase (5.63 μM) was incubated at room temperature with 0.37 mM H_2O_2, 0.16 M NaCl, and 0.2 M sodium phosphate, pH 6.1. The reaction was complete within a few seconds. Native myeloperoxidase (—); inactivated myeloperoxidase (---).

other significant changes in composition were noted, indicating that only oxidizable amino acids were affected during the inactivation process. It should be noted that at the high concentrations of enzyme necessary for amino acid analyses loss of the green color occurred in seconds, and a flocculent, white precipitate soon formed, indicating unfolding of the protein.

Discussion

Purification of human leukocyte myeloperoxidase, as described in the present paper, is a simple three-step procedure involving gel filtration and ion-exchange chromatography. The dialysis against low salt, which serves to precipitate elastase and cathepsin G, can be replaced by passage of the crude extract through a Sepharose–Trasylol column (Baugh & Travis, 1976). This substituted step allows for the purification of both elastase and cathepsin G which bind to the column while myeloperoxidase passes through unretarded with no loss in activity.

Table III: Effect of Autoinactivation on the Amino Acid Composition of Myeloperoxidase[a]

amino acid	native myeloperoxidase	oxidized myeloperoxidase
methionine	24	0
tryptophan	12	0
cysteine	24	0
tyrosine	24	21
histidine	12	6

[a] Myeloperoxidase was incubated as described under Methods until almost complete inactivation was attained. Acid hydrolyses and amino acid analyses were then performed.

The comparison of the purification procedure described here to those reported by other investigators has been difficult because of differences in the types of assays used (Zgliczynski et al., 1968; Harrison et al., 1977; Bakkenist et al., 1978). The only common denominator was the A_{430}/A_{280} ratio. This ratio, as determined by other investigators, has ranged from 0.76 to 0.83. The ratio of 0.83 has been quoted as that for crystalline enzyme (Schultz & Shmukler, 1964). The best ratio we have achieved is 0.71. While a low ratio may indicate excess A_{280} from contaminatng protein, a high value could indicate the presence of other proteins which absorb at 430 nm, such as hemoglobin. Incomplete separation of white from red blood cells could account for its prescence.

Even though the A_{430}/A_{280} ratio was not as high as that reported for other myeloperoxidases preparations, our NaDodSO$_4$–polyacrylamide gels and acid polyacrylamide gels contained only a single band. Other investigators whose enzyme preparations have higher ratios, nevertheless, report the presence of two proteins in NaDodSO$_4$ gels with varying molecular weights: 57 000 and 10 000 (Harrison et al., 197); 81 000 and 63 000 (Bakkenist et al., 1978); 62 000, 54 000, 38 000, and 14 000 (Olsson et al., 1972). In the second case, the possibility of the 81 000 component being a contaminant was noted by the authors. Our preparation also contained such a component, but it was removed by CM-cellulose chromatography. One team of investigators, however, has also obtained a preparation which yields a single band after NaDodSO$_4$–polyacrylamide gel electrophoresis of the reduced denatured sample (Desser et al., 1972). The molecular weight was reported to be 65 000, and it was proposed that the native enzyme was composed of two identical subunits.

The results obtained here suggest the organization of the myeloperoxidase protein. The enzyme apparently consists of two identical subunits held together by noncovalent bonds. The presence of two iron atoms per molecule has been reported (Schultz & Shmukler, 1964), indicating one iron atom per subunit. Each subunit appears to be more compact than expected from the molecular weight differences in a reduced vs. a nonreduced denaturing system. This may be due to intramolecular disulfide bridges.

The inactivation of myeloperoxidase appeared to be caused by oxidation of several susceptible amino acid residues. It is unlikely, however, that the extensive oxidation of myeloperoxidase occurred instantaneously since inhibition of α-1-PI could still be followed during the early stages of enzyme inactivation. Perhaps only a few amino acids are oxidized at

first, releasing the iron atoms. Further unfolding of the molecule may then leave other amino acids accessible to oxidative attack. Because of the necessity for use of relatively large amounts of enzyme for amino acid analysis, the reaction ws completed very rapidly, and, thus, only the final outcome of the inactivation reaction was determined.

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