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MYELOPEROXIDASE OF THE LEUKOCYTE OF NORMAL BLOOD

IV. SOME PHYSICOCHEMICAL PROPERTIES

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SUMMARY

The molecular weight of myeloperoxidase (donor:H_2O_2 oxidoreductase, EC 1.11.1.7) prepared from normal pig blood leukocytes was determined to be 139 000 by sedimentation and diffusion and to be about 130 000 by thin-layer gel chromatography. From the molecular weight and Fe content (0.069%) of the myeloperoxidase, it was concluded that the enzyme contains two atoms of iron per enzyme molecule. The amino acid composition of the myeloperoxidase preparation was analyzed and compared with that of myeloperoxidase prepared from leukocytes of normal human blood.

The light absorption spectra of the complexes of myeloperoxidase with cyanide, hydroxylamine and azide are given. In the reaction between myeloperoxidase and cyanide, two different complexes were formed at low and high cyanide concentration. The cyanide complex formed at lower cyanide concentration was found to contain only one cyanide per enzyme and a difference in the reactivity of two iron atoms was suggested.

INTRODUCTION

Based on the iron content of 0.074% (ref. 1) and the molecular weight of 149 000 (ref. 2), Agner[1] has concluded that myeloperoxidase (donor:H_2O_2 oxidoreductase, EC 1.11.1.7) prepared from pus of infected dog uteri contains two iron atoms per enzyme. From spectrophotometric observations Agner[3] has suggested that the H_2O_2 compound formed at lower peroxide concentrations contains only one peroxide molecule per enzyme molecule.

On the other hand, Schultz and Shmukler[4] have prepared myeloperoxidase from leukocytes of normal blood and reported the results of spectrophotometry and amino acid analysis carried out with the most purified enzyme. The iron content was determined as 0.093% but the molecular weight has not been reported. The purpose of this report is to describe some physicochemical properties of myeloperoxidase of

leukocytes of normal pig blood in order to clarify the characteristic features of the enzyme.

MATERIALS AND METHODS

Myeloperoxidase was prepared from leukocytes of normal pig blood by the method reported in a previous paper[5]. The concentration was calculated on the basis of a value of 95 $mM^{-1} \cdot cm^{-1}$ for the extinction coefficient at 430 nm (see text). The $A_{430\ nm}/A_{280\ nm}$ ratio for the enzyme used in this experiment was 0.7.

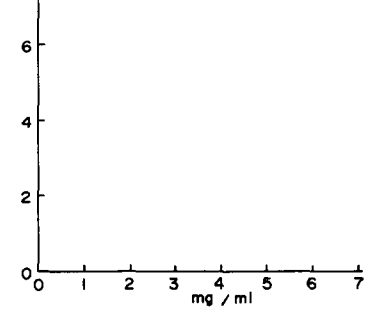
All other materials were obtained from commercial sources at the highest available states of purity. The reactions and the absorbance measurements were carried out in sodium–potasium phosphate buffer at 25 °C except as otherwise noted.

Measurements of sedimentation and diffusion were performed at 20 °C utilizing a Spinco model E (analytical) ultracentrifuge.

Atomic absorption was measured with a Hitachi Model 208 atomic absorption spectrophotometer. Amino acid analyses were performed with a Hitachi KLA-3B amino acid analyzer. Thin-layer gel chromatography was carried out by the method of Radola[6,7] utilizing a Pharmacia apparatus. The recording spectrophotometers used were Hitachi instruments, Types 124 and EPS-3T. The former instrument was used for the estimation of absorbance with an expanded scale.

RESULTS

The molecular weight of myeloperoxidase of normal pig leukocytes was determined by ultracentrifuge and gel chromatography experiments. The sedimentation coefficient ($s^0_{20,w}$) extrapolated to infinite dilution in the results of Fig. 1 was 7.9 S and the diffusion coefficient ($D^0_{20,w}$) was calculated from the results of Fig. 2 to be



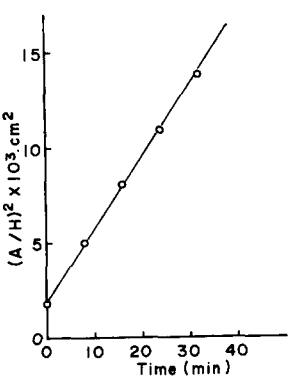


Fig. 1. Concentration dependence of the sedimentation coefficient of myeloperoxidase. Ordinate, sedimentation coefficient $s°_{20}$ calculated from enlarged drawing of the photographs. The centrifuge runs were carried out at 56 100 rev./min, 20 °C and the photographs were taken at an interval of 8 min.

Fig. 2. Calculation of the diffusion coefficient of myeloperoxidase. The graph was plotted according to the equation, $(A/H)^2 = 4\pi Dt$. The experiments were carried out at 15 220 rev./min, 20 °C and at a concentration of 1 mg myeloperoxidase/ml.

TABLE I

AMINO ACID COMPOSITION OF MYELOPEROXIDASE PREPARED FROM NORMAL BLOOD LEUKOCYTES OF PIG AND HUMAN

The values for pig enzyme are in terms of moles amino acid per enzyme. The molecular weight used was 139 000. The amino acids were determined after digestion in 6 M HCl at 110 °C. The analyses were carried out at 18, 24, 48, 72 and 96 h. Half-cysteine and methionine were analyzed after digestion by formyl hydroperoxide. A *n*-leucine solution was used as a marker for the calculation. A partial specific volume was calculated roughly to be 0.73 ml/g from this amino acid composition by the method of Schachman[9]. The values of human enzyme are those of Schultz and Shmukler[4] in terms of moles amino acid per 2 iron atoms.

Amino acid	*Species*	
	Pig	*Human*
Lys	33	22
His	13	8
NH_3	112	118
Arg	112	70
Asp	116	102
Thr	42	46
Ser	42	42
Glu	80	74
Pro	73	62
Gly	65	52
Ala	63	52
Cys	26	26
Val	60	34
Met	18	24
Ile	30	32
Leu	110	86
Tyr	19	16
Try	—*	13
Phe	48	34
Total	1062	946

* Not examined.

$5.1 \cdot 10^{-7}$ cm^2/s. Table I shows the amino acid composition of myeloperoxidase of normal blood of pig compared with that of human reported by Schultz and Shmukler[4]. From Table I the partial specific volume of myeloperoxidase of pig leukocytes was calculated approximately to be 0.73 ml/g. Insertion of $s^\circ_{20} = 7.9$ S, $D^\circ_{20} = 5.1$ F and $\bar{v} = 0.73$ ml/g in the Svedberg formula yields a molecular weight of 139 000 for the myeloperoxidase. Determination of molecular weight of the myeloperoxidase utilizing thin-layer gel chromatography was carried out according to Radola[6,7]. Fig. 3 shows the results of thin-layer gel chromatography with which the molecular weight of the myeloperoxidase was found to be about 130 000.

The iron content of the myeloperoxidase was analyzed by the atomic absorption of iron after wet-ashing the enzyme in a mixed solution of $HClO_4$ and HNO_3. The iron concentration of the myeloperoxidase solution was $2.3 \cdot 10^{-3}$ mg/ml. This enzyme solution had been dialyzed against several changes of deionized water. The dry weight of the enzyme solution was 3.46 mg/ml. From the iron concentration and dry weight of the enzyme solution the Fe content was calculated at 0.069%. Based on the iron content a minimum molecular weight of the enzyme was calculated at 80 000. Judged from $A_{430\text{ nm}}/A_{280\text{ nm}}$ ratio of 0.70 there is a possibility that the enzyme preparation

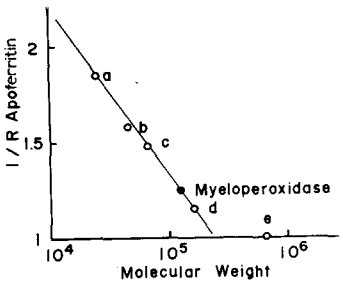


Fig. 3. Determination of molecular weight of myeloperoxidase by means of thin-layer gel chromatography on Sephadex G-200 (superfine, Pharmacia). Plate, 20 cm × 40 cm. Separation time, 4.2 h. Stained with bromocresol green. The gel was previously equilibrated with 0.01 M phosphate buffer (pH 7.5). Markers used were (a) chymotrypsinogen (25 000), (b) ovalbumin (45 000), (c) bovine serum albumin (67 000), (d) human serum γ-globulin (160 000) and (e) apoferritin (465 000).

contains about 10% impurities. The absorbance of the enzyme solution was 2.0 at 430 nm. Assuming that the enzyme contains two atoms of iron per enzyme the millimolar absorbance coefficient of the myeloperoxidase was then calculated at 95. These results are summarized in Table II in comparison with other analysts' data.

Agner[8] has reported that a compound of myeloperoxidase with HCN (KCN *plus*

TABLE II

SOME PROPERTIES OF MYELOPEROXIDASE FROM VARIOUS SOURCES

Sources	*Mol. wt* ($\times 10^4$)	*Iron content* (%)	*Iron atoms per enzyme*	$\frac{A_{430\text{ nm}}}{A_{280\text{ nm}}}$	ε_{mM} *at 430 nm in terms of 2 iron atoms*	*Partial specific volume*	s°_{20} (*S*)	D°_{20} (*F*)	*Reference*
(1) Normal pig leukocyte	(a) 13.9 (b) 13.0	0.069	2	0.70	95	0.73	7.9	5.1	This paper
(2) Human empyema fluid		0.1							8
(3) Rat chloroma tissue		0.07							10
(4) Dog pus of infected uteri	14.9	0.074	2	0.83	178*	0.731	7.93	4.8	2
(5) Normal human leukocyte	12.0**	0.093	(2)	0.80	92				4

(a) By ultracentrifuge. (b) By thin-layer chromatography with Sephadex G-200.

* This value of Agner is obviously twice the others. So, it might be pertinent to note his other results[1] which indicated that when myeloperoxidase was separated into two hemoprotein units the millimolar absorbance coefficient of the resultant hemoproteins lay between 42 and 50 in the Soret region.

** The molecular weight was calculated from the data of Schultz and Shmukler[4] assuming that the enzyme contains 2 iron atoms.

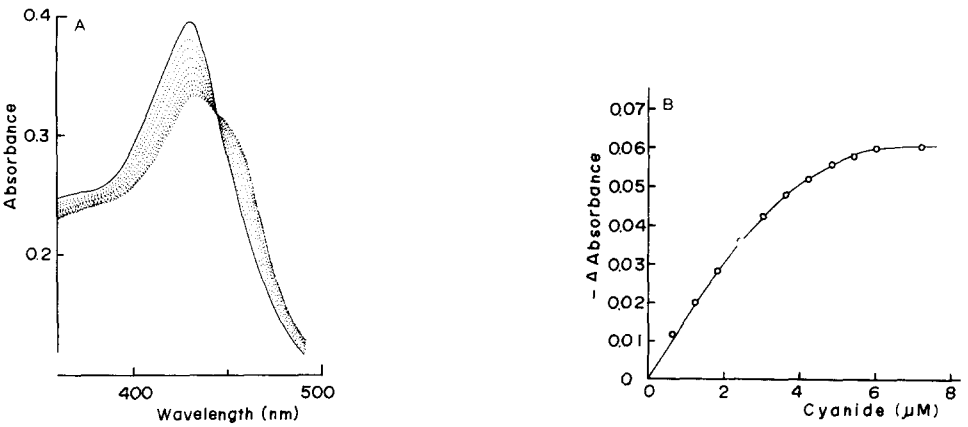


Fig. 4. (A) Titration of myeloperoxidase with cyanide. ———, 3.8 μM myeloperoxidase in 0.1 M phosphate buffer (pH 7.5). The dotted lines show the formation of CN^-–myeloperoxidase complex I by successive additions of 0.6 μM cyanide at each time. (B) Absorbance change at 430 nm was plotted against cyanide concentration.

equivalent HCl) has absorption bands at 458 and 634 nm. Fig. 4A shows the formation of a cyanide complex with absorption peaks at approx. 433 nm in the Soret region. This complex was formed in the presence of a small amount of cyanide. When the enzyme was titrated with cyanide the change in absorbance was proportional to the amount of cyanide added until it reached about the half maximal point (Fig. 4B). By extrapolation it was found that the enzyme combines with only one cyanide molecule in this cyanide-saturated state. This cyanide complex is similar to that reported by Morell[11]. The further addition of excess cyanide converted it to an another cyanide complex with absorption peaks approximately at 454 and 634 nm. This complex is similar to Agner's[8]. Fig. 5 shows absorption spectra of these two different cyanide complexes of myeloperoxidase.

A complex of ferric myeloperoxidase with H_2NOH was reported by Agner[8]. Fig. 6 shows the absorption spectrum of the H_2NOH–myeloperoxidase complex. Contrary

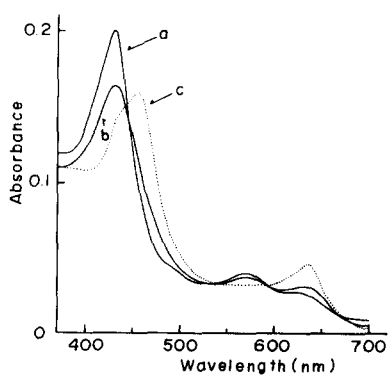


Fig. 5. Absorption spectra of two CN^-–myeloperoxidase complexes. Curve a, 2.1 μM myeloperoxidase in 0.1 M phosphate buffer (pH 7.5); Curve b, a + 4.8 μM KCN; Curve c, a + 4 mM KCN.

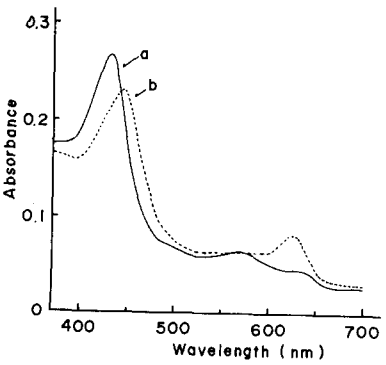


Fig. 6. Absorption spectrum of H_2NOH–myeloperoxidase complex. Curve a, 2.8 μM myeloperoxidase in 0.1 M phosphate buffer (pH 7.5); Curve b, 3.7 mM H_2NOH + a.

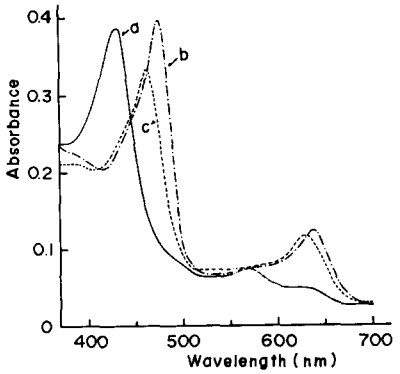


Fig. 7. Absorption spectrum of a complex between N_3^- and ferrous myeloperoxidase. Curve a, 4.1 μM myeloperoxidase in 0.1 M phosphate buffer (pH 7.0). Curve b, excess $Na_2S_2O_4$ was added to a. Curve c, b + 60 mM NaN_3.

to the Agner's results[8] an absorption maximum at 460 nm was not observed. Agner[8] also reported a ferrous myeloperoxidase–azide complex with absorption peaks at 460 and 615 nm. As can be seen in Fig. 7 in this experiment its absorption peak in the visible region was 630 nm instead of 615 nm. These results are collected in Table III. Agner[14] also reported a slight shift of the Soret band toward shorter wavelengths in the presence of NaCl, but the addition of 1 M NaCl did not cause such a change in our preparation.

TABLE III

THE ABSORPTION PEAKS OF MYELOPEROXDIASE DERIVATIVES

A number in parentheses shows a shoulder of the absorption spectrum.

Enzyme	*Wavelength (nm)*					*Reference*
Ferric	430	(500)	570	634*	690	5 and 8
Ferrous	475	(550)	585	637		5 and 8
Compound I	430					5
Compound I	430 (approx.)					12
Compound II	456			630		5
Compound II	458			625		8 and 12
Compound III	452		574	625.5		5
CO complex	468			634		5
CN^--complex I	433 (approx.)			634		This paper
CN^--complex II	454 (approx.)			634		This paper and 13
CN^--complex	458			634		8
CN^--complex	438			634		11
H_2NOH complex	443 (approx.)			624		This paper
H_2NOH complex	460			628		8
N_3^--complex	460			630		This paper
N_3^--complex	460			615		8

* A new observation in this paper. Absorbance of this peak differed from preparation to preparation.

DISCUSSION

The molecular weight of myeloperoxidase has been reported only for the enzyme prepared from the pus of infected dog uteri[2]. By means of independent analytical methods a reliable value has been found for the molecular weight of myeloperoxidase of normal pig leukocytes, which was similar to that of the pus enzyme reported by Ehrenberg and Agner[2]. As can be seen in Table II, there is no essential difference between these two sets of results except for the molar absorbance coefficient of the Soret peak. In this respect it may be of interest to compare the results with those of Schultz and Shmukler[4]. According to them a highly purified preparation of human leukocyte myeloperoxidase has an iron content of 0.093%. This is obviously higher than those of Agner's and our enzymes and it might be said that the human enzyme has a lower molecular weight. Although Schultz and Shmukler[4] did not describe the molar absorbance coefficient their data clearly indicate that the molar absorbance coefficient at 430 nm can be calculated as approximately at 46 on the basis of iron concentration. This value is very similar to ours.

Schultz and Shmukler[4] have determined the amino acid composition of myeloperoxidase of human leukocytes and suggested that the principal basic amino acid in myeloperoxidase is arginine. The present data also support their suggestion. In this experiment tryptophan was not specifically determined and it is impossible to conclude that the low $A_{430\ nm}/A_{280\ nm}$ ratio of our preparation is due to contamination by impurities. The results of ultracentrifuge and disc electrophoresis, however, indicate that such impurities are less than 10%.

In 1958, Agner[1] found that myeloperoxidase itself can be separated into two components in 50% pyridine solution and each of them has an absorption spectrum similar to the hemichrome of the original enzyme. Recently, the heterogenity of myeloperoxidase has been discussed by many workers[15–18]. Besides the well-known myeloperoxidase with a Soret maximum at 430 nm, myeloperoxidases which show a Soret maximum at shorter wavelengths have been reported by Schultz *et al.*[17] and Himmelhoch *et al.*[18]. It is obvious from the position and shape of the Soret spectrum that our preparation consists of only the former type of myeloperoxidase. However, the difference in reactivity between the two iron atoms in a molecule, which has been suggested by Agner[1], is confirmed by the following experiments.

From the results of Figs 4 and 5 it is obvious that at least two different cyanide–myeloperoxidase complexes are formed. Of special interest is the result that one of the two iron atoms has a particularly high affinity for cyanide. Since myeloperoxidase contains two heme groups[1] the functional difference between the two iron atoms must be a key point in the analysis of the mechanism of this unusual peroxidase. From spectrophotometric analyses Agner[3] suggested that at low H_2O_2 concentration the peroxide reacts with one of the two iron atoms. Although his suggestion had not been confirmed by the stoichiometric reaction the present cyanide data are consistent with Agner's idea. From Agner's finding[1] that myeloperoxidase can be separated into two different hemoprotein subunits, the two heme groups appear to have inherently different reactivities for hydrogen peroxide and cyanide. Interaction between these two sites, however, cannot be excluded at the moment and the situation may be somewhat similar to the case of cytochrome oxidase.

Agner[8] reported a cyanide complex of myeloperoxidase with absorption bands

458 and 634 nm while Morell[11] in his earlier experiment observed a cyanide complex of myeloperoxidase with absorption bands at 438 and 634 nm. As can be seen in Table III it is likely that cyanide–myeloperoxidase Complex I in the present study corresponds to Morell's complex and cyanide–myeloperoxidase Complex II to Agner's. Recently, Newton *et al.*[13] have reported a cyanide complex with the same absorption bands as those of cyanide Complex II. The formation of cyanide Complex II is completed only at very high cyanide concentration and it remains unknown whether the second cyanide reacts with an open heme iron or other nonmetallic sites.

It was reported by Agner[8] that the H_2NOH–myeloperoxidase compound has absorption bands at 460 and 628 nm and that an azide–ferrous myeloperoxidase compound has absorption bands at 460 and 615 nm. Table III shows that our results are slightly different from Agner's. The differences might reflect species differences.

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