MYELOPEROXIDASE VIII: SEPARATION INTO TEN COMPONENTS BY FREE-FLOW ELECTROPHORESIS

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The physical and chemical properties of myeloperoxidase have been difficult to explain in terms of its known structure as a hemeprotein containing two hemes per molecule. The reaction of the enzyme with hydrogen peroxide, with the chloride ion, and precipitation with methanol-pyridine lead to the proposal that both hemes were equal (Agner, 1958) and another investigator (Paul, 1963) to suggest unequally reactive hemes. The reaction with ferrous ion and oxygen (Schultz and Rosenthal, 1959), which inactivates the enzyme, resulted in the finding by Rosenthal and Schultz (1965) that ferric ion and hydrogen peroxide were possible intermediates and gave rise to the proposition that at least two kinds of prosthetic groups were present and that they may not both be hemes. Two components were observed in acrylamide gel and separated on Sephadex (John and Schultz, 1965). A. Ehrenberg (1962) suggested that either the hemes were surrounded by different microenvironments or were of two kinds on the basis of the electron spin resonance spectrum. The present report offers evidence for the presence of at least ten components separated in 6M urea in a Brinkman Free-Flow electrophoresis apparatus. These components can be divided into two groups on the basis of light absorption in the Soret region; the one group

characterized by maxima close to that of the native enzyme (430mμ), and the other about 20-25 millimicrons toward the shorter wave lengths. The remarkable distinction between the two groups is that the former shifts to longer wave lengths (about 40 millimicrons) on reduction and the latter bleaches on treatment with hydrosulfite, very unlike any known heme. In addition, the amino acid analysis of each of the components indicate that each are discrete proteins of different amino acid composition. The findings reported here offer a new basis for explaining the peculiar properties of myeloperoxidase which led one investigator (Ehrenberg, 1962) to conclude after comparing it with other hemeproteins that the neutrophilic peroxidase was a protein of the "most unusual physical and chemical properties."

METHODS AND MATERIALS

The myeloperoxidase was prepared by the procedure of Schultz and Shmukler (1964) except that the final purification was carried out in a Brinkman Free-Flow in 0.01M phosphate buffer (pH 7.6) and the material of A_{430}/A_{280} of 0.83 was collected and combined. Forty-seven mgs. were dissolved in 1.7 ml on 6M urea-Tris-glycine buffer (pH 8.6; 7.4mM Tris-0.575M Glycine). The electrode buffer was three times more concentrated than the chamber buffer. Electrophoresis was conducted at a current of 90mA at 1800V, a buffer rate at 1 ml/hr. and sample application at the same rate. Total running time was four hours.

After the four hours of running time, the contents of each of the 48 collection tubes were examined on a Beckman DB at 280mμ and at the Soret maximum by scanning the range 400-435mμ. Peroxidase was assayed by the procedure previously described (Schultz and Shmukler, 1964). Peak tubes were combined, dialyzed and in those cases where Spectra were

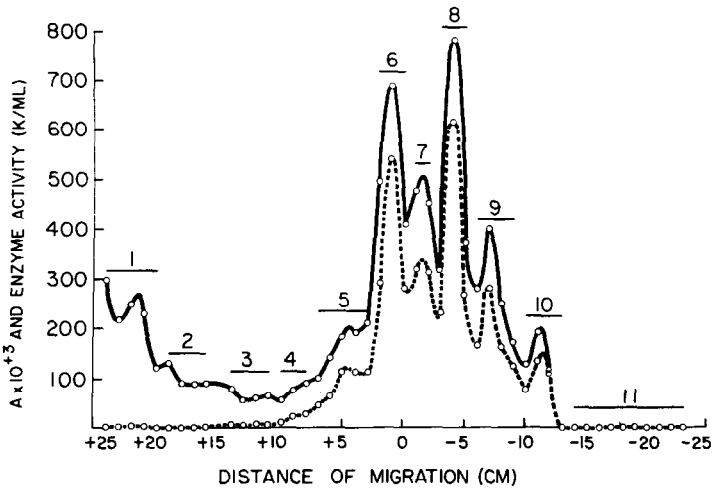

Figure 1. The distribution of peroxidase activity and heme absorption at the Soret maxima following electrophoresis of myeloperoxidase in 6M urea tris-glycine buffer pH 8.6 on a Brinkman Free-Flow electrophoresis apparatus. Solid lines are absorption at Soret maxima; dotted lines indicate peroxidase activity. Numbers above horizontal lines are peaks referred to in the text and each line covers the collection tubes combined and included in that fraction. Amino acid analysis of each fraction are recorded in Table I and spectrophotometric data in Table II.

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scanned, a Process and Instrument Recording Spectrophotometer in a 3 ml cuvette with 10 mm light path was used.

Amino acid analyses were carried out on the dialyzed, lyophilized samples dissolved in 6N HCl and hydrolyzed for 24 hours at 105° in sealed tubes. The procedure of Spackman et al., (1958) was followed using a Phoenix Amino Acid Analyzer with the accelerated column.

Figure 1 and Table 1 show the separation of at least ten components and the amino acid analysis of each. The components may be divided into two general groups; those with Soret maxima at 410-415mμ and those at 425-430mμ as in Table II.

AMINO ACID COMPOSITION[1] OF COMPONENTS OF MYELOPEROXIDASE

TABLE I

Amino Acid	Components										Native Enzyme
	1	2	3	4	5	6	7	8	9	10	
	(Micromoles x 10^3)										
Lys	10	21	18	39	29	33	38	44	29	35	11
His	11	07	04	08	15	12	15	12	08	09	4
Arg	09	27	21	31	82	134	13	127	112	53	35
Asp	84	101	66	76	92	181	141	173	142	95	51
Thr	33	39	25	33	37	71	57	69	57	36	23
Ser	47	76	57	107	48	84	72	74	68	52	21
Glu	79	72	56	96	78	148	117	124	121	96	37
Pro	21	29	*	*	13	79	45	81	80	33	31
Gly[2]	124	85	76	132	64	111	84	91	88	65	26
Ala	28	43	37	50	49	97	76	83	80	48	26
1/2 Cys	*	27	16	14	11	20	05	04	13	*	13
Val	09	27	17	19	17	55	35	53	41	16	17
Met	*	05	*	*	07	39	20	12	**	17	12
Ileu	21	29	23	24	35	53	41	56	**	21	16
Leu	59	61	49	53	93	160	132	142	**	83	43
Tyr	*	07	11	05	15	39	19	13	**	17	8
Phe	28	24	21	23	40	63	48	57	**	37	17

[1] All values refer to micromoles of amino acid found in the aliquot taken for analyses. Each represents a single run on a 24 hour hydrolysis in 6N HCl at 105°. Values for the native enzyme (Schultz and Shmukler, 1964) are recorded in the last column.

[2] Gly values are high probably due to glycine buffer.

* Absent or too little to analyze.

** Loss due to malfunction of machine.

On reduction with hydrosulfite, the anodic components bleach (*) without the appearance of a new maxima, in contrast to the more cathodic protein which undergoes a shift to longer wave lengths characteristic of native myeloperoxidase (Schultz and Shmukler, 1964). Also characteristic

Table II
Soret Maxima (Oxidized and Reduced)
Each figure represents Soret maxima in mμ

	Components									
	1	2	3	4	5	6	7	8	9	10
	Soret (mμ)									
Oxidized	407	408	411	409	420	424	427	425	426	428
Reduced	*	*	*	*	470	471	472	472	470	471

is the amino acid composition which is remarkably distinct for each separated fraction. The ratio of arginine to lysine, which in the native enzyme is 35 to 11, varies from unity in the anodic components, to 2-3 to 1 in the cathode proteins and a complete reversal in No 7, to 1 to 3. The native enzyme has 12 residues of methionine to 11 of lysine; but it can be seen in Table II that methionine is found principally in the cathodic components, while cystine is absent or found in small amounts in at least half of them. There is sufficient variation beyond the error of the procedures to allow that these are different proteins.

DISCUSSION

The criteria of purity of myeloperoxidase is based on the reports of the ratio of the A_{430}/A_{280} (0.83) of the crystalline enzyme (Agner, 1958), its homogeneity in the ultracentrifuge (Ehrenberg and Agner, 1958), the report of amino acid analysis and the confirmation of its spectrophotometric constant were confirmed (Schultz and Shmukler, 1964). Two other laboratories by other methods of preparation have also prepared material of the

A_{430}/A_{280} equal to 0.83 - 0.85 (Rohrer et al., 1966) (Newton et al., 1965). On this basis one must consider that the purity of the enzyme in the present experiments is comparable to that which is recognized as pure myeloperoxidase.

It must be kept in mind that myeloperoxidase is available in very limited amounts from various sources of white cells. The results obtained here are offered as evidence of the fact that myeloperoxidase, hitherto considered a one or two component system, can be dissociated by urea in an electric field into at least ten components. Limitations of material at the present time do not allow for more complete characterization of these components through repeated amino acid analysis, ultracentrifugal molecular weight studies and other physical constants that will be reported at a later date. A study, however, of the nature of the material absorbing in the Soret region was carried out on the most anodic protein, component number one, to determine whether or not it was a heme. There was no evidence of a pyridine hemochromogen so characteristic of the native enzyme (Schultz and Shmukler, 1964); in fact, reduction in pyridine-sodium hydroxide showed no alteration in spectrum.

The contribution of these data to the nature of myeloperoxidase in the native state is limited to the speculation that the anodic and cathodic components may be part of a system wherein the native enzyme consists of one each anodic and one each cathodic protein held together by ionic linkage, and that in urea the dielectric is sufficiently low to prevent their separation by gravitational forces, but are separated by high electric field strengths. Studies by Dr. Stelos at Hahnemann of the native enzyme at pH 8.6 with or without 6M urea showing an undissociated homogeneous single component in each case support this contention. The relation of these findings to the activity of the enzyme is difficult at the present time because of the minor

alteration in structure that does take place in the presence of urea, which is evident from the abnormally high absorbancy at 280mμ in respect to the absorbancy at the soret. In urea this ratio falls to 0.70 even after removal of the urea; and lower values are obtained in the active fractions described here.

It is quite likely that some changes have taken place in confirmation of each protein following separation. In this respect the question whether these are isozymes or actually part of a system wherein maximum activity requires the initial intact complex remains open. Nevertheless, the presence of a new kind of prosthetic group has been demonstrated as suggested from earlier studies (Rosenthal and Schultz, 1965).

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