THE PROSTHETIC GROUP OF MYELOPEROXIDASE

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1. Introduction

In general, when hemoproteins are treated with acidified acetone, the protein is precipitated, while the heme remains in solution. Exceptions are cytochrome *c*, lactoperoxidase, and myeloperoxidase; the linkages between heme and protein in these instances being covalent. While the nature of the linkages in cytochrome *c* and lactoperoxidase have been characterized, that present in myeloperoxidase remains unknown. Previous studies have, however, indicated that the linkage is not the heme c type [1] and that low yields of the prosthetic group are obtained with reagents which readily cleave ester bonds such as methanolic HCl [2] and HI in acetic acid [3]. In this report we consider the possibility of an amide bond.

To differentiate between an ester and an amide linkage in myeloperoxidase, we have investigated the lability of the bond toward sodium methoxide. In comparing our results with those Morrison [4] obtained with lactoperoxidase (ester bond), we conclude that the bond in myeloperoxidase is significantly more stable, and therefore amide.

In addition, examination of the products of methoxide and hydrazine cleavage show that the derivatives of myeloperoxidase heme obtained by such hydrolysis are very closely related to protoheme IX. A preliminary account of portions of this study has been reported [5].

2. Materials and methods

Myeloperoxidase was purified from normal human leucocytes [6]. The enzyme used in the experiments possessed the RZ above 0.8.

2.1. *Reactions of the enzyme with sodium methoxide in methanol*

Three aliquots of 5 mg myeloperoxidase were homogenized in 1 ml of methanol. 1 ml of sodium methoxide in methanol was added slowly, with constant stirring to each suspension; a 1 M aliquot to the first, 2 M to the second, and 4 M to the final suspension. The reaction was allowed to proceed at room temperature for 20 min. The reaction mixture was acidified with 2 N HCl and extracted twice, each with 2 ml ether and the extract was washed twice with 4 ml of water. Finally, the extract was dried by anhydrous sodium sulfate and evaporation. Three additional samples of each of myeloperoxidase were treated with 2 M sodium methoxide in methanol for 10 min, 20 min and 30 min, respectively. The rest of the procedure was the same as the previous one.

2.2. *Reactions of enzyme with hydrazine in acetic acid*

5 mg of myeloperoxidase were suspended in 1 ml of 2.5% hydrazine in acetic acid solution, and heated at 105°C under an atmosphere of nitrogen in a sealed vial for 30 min. The solution was cooled at room temperature, and neutralized with 10% sodium acetate. The red fluorescent porphyrin was extracted twice, each with 2 ml of ether, and the ether extract was washed twice with 2 ml of water. A few crystals of anhydrous Na_2SO_4 were added to ether extract and the ether solution was transferred to a beaker and evaporated to dryness.

2.3. *Esterification of porphyrin*

The porphyrin was converted to methyl ester in methanol—sulfuric acid (19:1) solution at room temperature for 16 h [7]. The methyl esters of the porphyrins were purified by paper chromatography. The

porphyrins were dissolved in chloroform, and streaked on a Whatman No. 1 paper (18 X 18 cm). The chromatography was developed ascendingly by the solvent system of propanol—kerosene (1:5). The porphyrins were located by red fluorescence, and the major area was cut and eluted by chloroform. The eluate was dried by evaporation.

2.4. Characterization of porphyrin and heme

The porphyrins and heme were characterized spectrally and chromatographically. The methyl esters of porphyrins were identified on Whatman No. 1 paper (18 X 18 cm) and the solvent systems: kerosene—tetrahydropyran—methyl benzoate (5:1.4:0.35), water—acetonitrile—n-propyl alcohol—pyridine (3.8:1:2:0.5) [8], n-propanol—kerosene (1:5), n-propanol—isooctane (1:5) [9], and toluene with Dow-Corning silicone NO. 550 as stationary phase. Spectrally, the porphyrins were scanned in ether and chloroform solvent between 700 and 350 nm. The heme cleaved from enzyme was dissolved in a solution of pyridine—0.05 N NaOH (1:1) with a few crystals of sodium hydrosulfite. The pyridine ferrohemochrome was measured on spectrophotometer.

3. Results

3.1. Characterization of heme and its linkage to apoprotein

The effect of the concentration of methoxide on the cleavage rate is shown in fig.1. Higher yield of
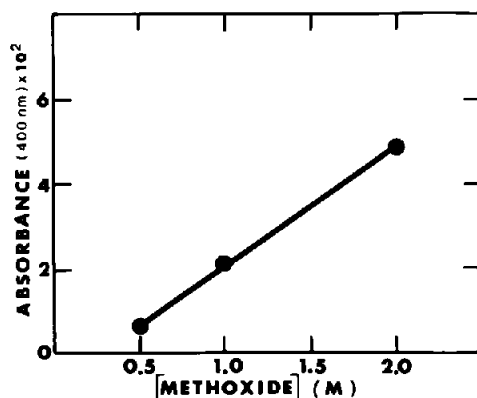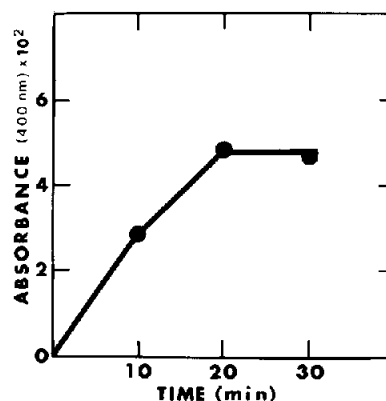

Fig.2. Time-course of bond cleavage between heme and apoprotein. The condition is described in Methods.

heme was obtained, while the methoxide concentration was increased in the hydrolysis medium. The cleavage of the bond between heme and apoprotein was not instantaneous. As illustrated in fig.2, maximal cleavage required at least 20 min under these experimental conditions. Spectral determination of the product of sodium methoxide cleavage showed it to be almost indistinguishable from protoheme in table 1.

3.2. Characterization of porphyrin

Spectral study of the methyl ester of porphyrin isolated from myeloperoxidase showed an etio-type spectrum in ether solvent in fig.3. The absorption maxima of the methyl esters of hydrazine product corresponded to that of dimethyl ester of protoporphyrin IX in both ether and chloroform in table 2.

Fig.1. Effect of methoxide concentration on heme-apoprotein bond cleavage rate. The condition is described in Methods.

Table 1
Absorption maxima of pyridine ferrohemochromes of enzymes and hemes

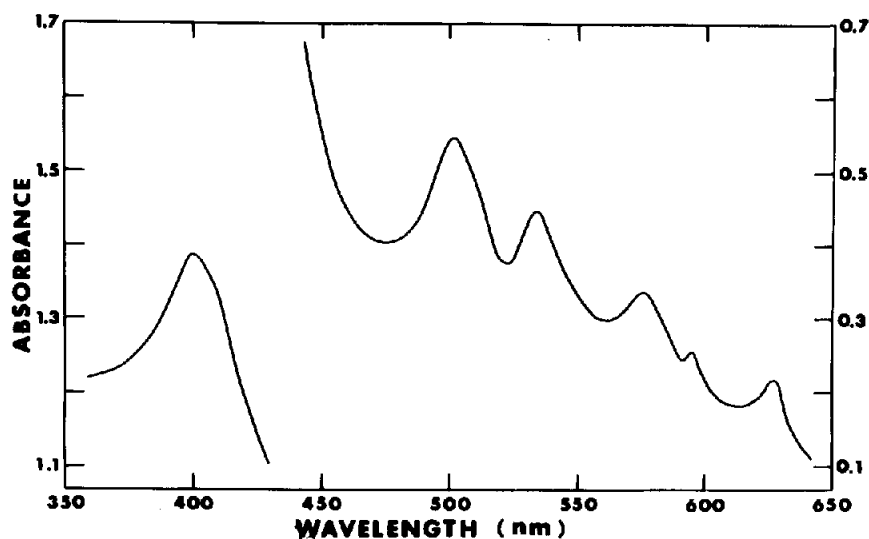| Enzyme or heme	α-band (nm)	β-band (nm)	γ-band (nm)
Myeloperoxidase	586	—	435
Product of sodium methoxide	562	562	418
Protoheme	556	525	419

Fig. 3. Absorption spectrum of porphyrin isolated from myeloperoxidase by hydrazinolysis. The porphyrin was methylated and measured in ether.

In chromatographic studies, the R_f values of the methyl esters of porphyrin in five solvent systems are compared in table 3. The methyl ester of the product of hydrazine indicated the similarity to dimethyl ester of protoporphyrin IX.

4. Discussion

The rate of bond cleavage and the concentration of sodium methoxide shown that the linkage of heme in myeloperoxidase is more stable than that in lactoperoxidase [4]. The identity of this linkage as amide bond would explain the resistance to cleavage at the expense of methanolic HCl [2] and HI in acetic acid [3]. An amide bond for heme has not previously been described, although other prosthetic groups are known to be attached in this manner.

The pyridine ferrohemochrome spectrum of myeloperoxidase, which is very similar to that of heme a [10] is generated by electrophilic substituents on opposite pyrrole rings [11]. The small differences

Table 2
Absorption maxima of methyl esters of porphyrins

Porphyrin	Solvent	Soret	IV	III	II	I
				(nm)		
Protoporphyrin	ether	402	502	537	578	633
Product of hydrazine	ether	400	500	537	576	632
Protoporphyrin	chloroform	406	505	541	577	632
Product of hydrazine	chloroform	406	505	542	572	633

Table 3
R_f values of methyl esters of porphyrins in paper chromatography

| Porphyrin | Solvent systems | | | | |
	KTM[a]	WAPP[b]	n-proponol–kerosene	n-propanol–isooctane	Toluene
Product of hydrazine	0.58	0.09	0.73	0.80	0.84
Protopor-phyrin	0.59	0.09	0.73	0.80	0.84
Meso-porphyrin	0.70	0.10		0.85	
Hemato-porphyrin	0.19	0.40			

[a]KTM; kerosene, tetrahydropyran and methyl benzoate.
[b]WAPP; water, acetonitrile, n-propanol and pyridine.

between the pyridine ferrohemochrome of myeloperoxidase and heme a are probably due to the presence of an additional vinyl group in the myeloperoxidase heme, where an α-hydroxyalkyl group is present, in heme a at position 2. Chromatographic examinations of the cleaved product have confirmed the absence of this heme a character. This argument alone would suggest that the electrophilic group on the myeloperoxidase heme resides on either ring III or IV; these rings bear the methyl and propionic side chains in protoheme.

Protoporphyrin presumably arises under conditions of hydrazine cleavage by Wolf-Kishner reduction, while acetal formation can account for the spectral properties of the methoxide-cleaved heme. Treatment of myeloperoxidase with $NaBH_4$ results in a hemochrome showing maxima at 418, 523, 557 nm, compared to protoheme, 419, 525 and 556 nm [12]. The simplest explanation of these three findings is that a single formyl group resides on ring III or IV, implying that 2,4-divinyl-8-(5)-formyl-deuteroporphyrin IX is the heme of myeloperoxidase. This conclusion is at variance with that of Nichol et al, who concluded that the carbonyl substituent of the myeloperoxidase heme is present on a two-carbon side chain [2].

Further studies on the heme structure and linkage are in progress.

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