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THE HAEMATIN PROSTHETIC GROUPS OF SOME ANIMAL PEROXIDASES

I. THE PREPARATION AND PROPERTIES OF AN ETHER-SOLUBLE HAEMATIN FROM MILK PEROXIDASE

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SUMMARY

A study of the pyridine haemochrome of milk peroxidase shows that the prosthetic haematin group contains a strongly electrophilic substituent conjugated to the porphyrin ring. This substituent is labile to strong alkali and to certain adsorbents. The haematin is co-valently bound to the apoenzyme through ester or amide bonds. This linkage can be broken by alkali and by either hydrogen iodide or hydrogen bromide in acetic acid. Only the latter reagent yields a haematin which retains the characteristic electrophilic group. The nature of this group is as yet unknown.

INTRODUCTION

Whereas the many peroxidases so far investigated in plants have protohaematin as their prosthetic groups, two of the most-studied peroxidases of mammals, milk peroxidase and leukocyte peroxidase, are known to have prosthetic groups different both from protohaematin and from each other. The spectra of milk peroxidase and its derivatives[1-5] indicate that the prosthetic group of this enzyme is the iron complex of a porphyrin substituted on a β-pyrrole position by at least one strongly electrophilic side chain. Again, reduced leukocyte peroxidase has a band at 637 mμ (see ref. 6), far to the red of those of milk peroxidase[5] (565 mμ) and horse-radish peroxidase[7] (556 mμ). Its pyridine haemochrome absorption band at 590 mμ (see ref. 8) also lies well to the red of those for the other peroxidases (see later). FOULKES, LEMBERG AND PURDOM[9] have suggested that the prosthetic group of leukocyte peroxidase is chemically related to the pseudohaematin of choleglobin, while NICHOLLS[10] has drawn attention to the similarity of the spectrum of leukocyte peroxidase and its derivatives to that of sulfmyoglobin and sulfcatalase.

The haematin groups of both milk peroxidase[4] and leukocyte peroxidase[6] are known to be co-valently bound to the apo-protein. The present paper deals with the preparation of an ether-soluble haematin from milk peroxidase using hydrogen

bromide in acetic acid as the splitting agent. It also describes some of the properties of the soluble haematin and compares them with those of the unsplit haemoprotein. Recent brief communications by MORRISON AND HULTQUIST[11] describe the preparation from milk peroxidase of an ether-soluble meso-type porphyrin using hydrogen iodide in acetic acid and of an ether-soluble proto-type haematin using alkaline hydrolysis. He suggests that the prosthetic haematin contains two vinyl groups and is "a derivative of protohaematin". However, the spectral data on the enzyme mentioned above indicate the presence of one or more electrophilic groups with a total electron-attraction considerably greater than that of the two postulated vinyl groups. Thus both the procedures used by MORRISON, whilst informative with regard to the haematin-protein bonds, destroy or substantially alter the electrophilic properties of the characteristic group or groups of milk peroxidase haematin. This disadvantage does not arise when HBr is used as the splitting agent.

Although MORELL[4] was previously unable to split the haematin group from milk peroxidase, removal of the iron atom from the haematin produced a porphyrin-protein compound with a spectrum similar to that of monoacetyldeuteroporphyrin. The present work confirms the order of strength of the electrophilic substituent but does not identify it.

Further papers will deal with details of chemistry of milk peroxidase haematin and its derivatives and with studies of the haematin group of leukocyte peroxidase.

MATERIALS AND METHODS

Hydrogen bromide: The reagent used was B.D.H. 50 % (w/v) in glacial acetic acid.

Solvents: All solvents except ether were distilled prior to use. Ether, not containing reducer, was used as supplied.

Spectroscopy: Unless otherwise stated light-absorption data were obtained using a Hilger "Uvispek" spectrophotometer, the wavelength setting of which was standardized according to the hydrogen lines at 486.1 mμ and 656.3 mμ.

EXPERIMENTAL

Preparation of milk peroxidase haemoprotein

The haemoprotein was purified essentially according to the method of MORRISON, HAMILTON AND STOTZ[12]. In the present work the starting material was cheese whey obtained at the normal stage of "wheying off" during the manufacture of cheddar-type cheese. Batches were of about 270 gallons requiring approx. 3 kg "Amberlite" CG-50 ion-exchange resin. After adsorption of the enzyme the resin was extensively washed with tap water both by decantation and in a polythene chromatography tube 36 in long by 6 in in diameter. After elution and dialysis against cold tap water the enzyme was chromatographed on a second column (24 in of resin by 3 in in diameter) using the same type of resin. Both lots of resin were in the ammonium form. The haemoprotein product, as used for preparation of the milk-peroxidase haematin, had a ratio, absorbance at 412 mμ (Soret band)/absorbance at 280 mμ (protein absorption), of 0.60–0.77. MORRISON *et al.*[12] obtained a value of 0.80 for this ratio using a very highly purified enzyme solution.

Splitting of the haematin from the apo-protein

Successful splitting of the haemoprotein as judged by a haematin product soluble in ether was achieved by the following method. Freshly prepared enzyme was precipitated in the cold from its solution in dilute phosphate buffer with ammonium sulphate at 80% saturation. After centrifuging in the cold the haemoprotein precipitate was drained and dissolved in glacial acetic acid at room temperature. The resultant precipitate of ammonium sulphate was centrifuged off and washed once with glacial acetic acid. The supernatants from the centrifuged washings were added to the main solution of haemoprotein. An equal volume of hydrogen bromide in glacial acetic acid (50% w/v) was added and the mixture set aside in a stoppered flask in the dark for 2 h. It was then shaken in a separating funnel with a mixture of ether and water, the haemin separating into the ether layer.

A higher yield of haematin was achieved when the haemoprotein is dissolved in acetic acid, as described above, before adding the HBr in acetic acid than when dissolved directly in HBr-acetic acid. The recommended procedure aids solubility of the haemoprotein. In earlier experiments before refrigeration facilities were available, the enzyme was precipitated with acetone at about 10°. In some of these experiments no ether-soluble haematin was obtained although the solution of haemoprotein in acetic acid was quite clear. Presumably denaturation of the haemoprotein by acetone prevented access of the HBr to the bond or bonds linking haematin to protein.

In addition to the ether-soluble haematin the HBr splitting procedure also produced some violet-coloured pigment, insoluble in ether or amyl alcohol and having a strong, broad absorption with a maximum at about 590 mμ. This spectrum in 20% HCl (w/v) resembles that of mesobiliviolin. The following experiments indicate that this by-product is probably derived from the enzyme haematin. Concentrated HCl (0.2 ml) was added to 3.0 ml of the haemoprotein solution in acetic acid. Within 1 h at room temperature a rose-pink colouration was evident by transmitted light becoming bluer with time. Water hastened the reaction. Study of the visible and Soret spectra also indicated a complex reaction. When the haemoprotein was dissolved in concentrated HCl no haematin remained after several days at room temperature as judged by the spectrum or the benzidine–H_2O_2 test. It is, however, possible that the violet compound is produced from a second haematin component of the preparation.

The yield of the violet by-product varied in different preparations from trace amounts to considerable quantities. Production of this compound seems to be a property of the preparation rather than the splitting reagent. This is suggested by an experiment in which two separate preparations were split on the same day with the same batch of reagent. No detectable by-product resulted in one case, the other giving a considerable quantity.

The ether-soluble haematin obtained from the haemoprotein by treatment with HBr–acetic acid did not give rise to violet by-product after further standing in HBr–acetic acid nor was it affected by solution in a mixture of ether and concentrated HCl (single phase) for four days.

The prosthetic group can also be split from the protein with hydrogen iodide in acetic acid as has also been found by MORRISON[11]. In this case a leuco compound is produced which, on autoxidation, gives a porphyrin similar to mesoporphyrin in spectrum and HCl number. This reaction apparently reduces the electrophilic side

chains of the original haematin and is therefore of little value in studying their nature.

An ether-soluble haematin has also been achieved after alkaline hydrolysis in confirmation of the finding of MORRISON[11]. In our experiments hydrolysis at 85° for 45 min in 1 N NaOH was used. After this treatment the haemochrome in pyridine–water had an absorption maximum at 559 mμ which indicates an alteration in the characteristic electrophilic group. However, this method is of value in showing that the haematin is probably bound to the protein by ester or amide linkages

Several attempts failed to split the enzyme using the silver sulphate procedure of PAUL[13] which splits the thio-ether bonds linking haematin to protein in cytochromes. In our experience the method of BARRETT AND KAMEN[14] in which mercurous sulphate in acetic acid is used is superior in splitting cytochrome *c* to PAUL's procedure. This reagent, however, also failed to split milk peroxidase either before or after digestion with pepsin and trypsin. These results indicate that the haematin of milk peroxidase is not bound to the protein by thio-ether bonds but by another co-valent linkage, a conclusion also reached on similar grounds by MORRISON AND HULTQUIST[11].

Purification of the ether-soluble haematin

Separation of much of the haematin from a yellow lipid and possibly other material has been achieved using distribution between the aqueous acetone–HCl and acetone–light petroleum phases as used by KIESE AND KURZ[15] for the separation of haematin *a* and protohaematin. The yield of lactoperoxidase haematin in the lower phase is much greater if it is pretreated overnight by standing in a single phase mixture of ether and concentrated HCl containing minimal ether. The purification step did not alter the haematin as judged by its haemochrome in pyridine–water. After washing out most of the HCl and evaporation of the ethereal solution to dryness the haemin was now found to be completely insoluble in light petroleum (60–80°). A further purification of the haemin was achieved by precipitation of some material in acetone at −15°. The haemin then had a specific extinction (absorbance at 543 mμ/mg haemin) of 3. Comparison with protohaemin (specific extinction at 540 mμ = 15) indicates that the peroxidase haemin is impure or that it has a remarkably high molecular weight.

Other methods of purifying the haemin have not been successful owing to its instability towards alkali or to certain adsorbents. Chromatography on cellulose, Celite 512, or silicic acid columns produced some spectroscopically altered haemin. The haemin could not be esterified for chromatography because it was altered both by acid methanol and by diazomethane[16].

Spectroscopic studies

Although the α-band of the pyridine haemochrome of milk peroxidase has been published by a number of workers[1–3,5,11] at about 565 mμ, the present study using the Hartridge Reversion Spectroscope has shown that this result is only the initial value obtained using pyridine–0.1 N NaOH (1:3, v/v). With time there is a drift in the absorption maximum to shorter wavelengths reaching 556–558 mμ in about 10 min. This effect cannot be reversed by neutralizing the NaOH. When water is used instead of the alkali solution, however, there is a drift to longer wavelengths reaching 569–571 mμ within 1 h. Incubation of the enzyme in 0.1 N NaOH alone for 10 min followed by the addition of pyridine (to 25%, v/v) and dithionite gave

a haemochrome maximum at 563 mμ. This experiment suggests that the effect of alkali on the haemoprotein is hastened by the presence of pyridine.

Study of the pyridine haemochromes of the soluble haematin using the same reagent mixtures as above gave the following results. In pyridine–alkali the same drift of the maximum to shorter wavelengths was found. In pyridine–water values of 566–568 mμ, stable for at least 1 h, were obtained. In no case did the value in pyridine–water go to higher wavelengths as with the haemochrome of the intact haemoprotein. This measure of agreement between the haemochromes of the derived haematin and the intact haemoprotein indicated that the ether-soluble haematin would be of considerable value in chemical studies designed to elucidate the structure of the prosthetic group of the enzyme.

Comparison of the Soret bands of the haemoprotein (aqueous solvent) and the ether-soluble haemin (ether solvent) showed an interesting divergence. In the Soret region both oxidized and reduced enzyme give single Soret peaks whereas the soluble haemin shows maxima at 370 mμ and 415 mμ. This phenomenon has been the subject of a separate study described in the accompanying paper[17].

This latter investigation has revealed two spectroscopic properties of haematins relevant to the structure of milk peroxidase haematin. (a) Two Soret maxima are normally exhibited by haemins when the solvent is relatively non-polar as in ether or benzene. (b) The position of the Soret maximum at longer wavelength (S band) and the ratio of the extinctions of the S to S_1 bands (for nomenclature see CLEZY AND MORELL[17]) are dependent upon the electrophilic character of conjugated substituents.

In milk peroxidase haematin the position of the S band at 415 mμ indicates the presence of a conjugated electrophilic group. The ratio of 1.35 for the Soret extinctions, S band/S_1 band, suggests the presence of a single conjugated electrophilic substituent (see Table I, CLEZY AND MORELL[17]).

Formation of porphyrin from the ether-soluble haematin

Two methods have been used to convert the haematin to porphyrin.

1. To 7 mg haematin in 10 ml acetic acid were added 30 ml HBr–acetic acid and 100 mg finely ground $FeSO_4 \cdot 7H_2O$. After 15 min at room temperature the conversion to porphyrin was complete. Virtually only one porphyrin resulted and on immediate transfer to ethereal solution had the following band positions: band I, 643 mμ; band II, 582 mμ; band III, 552 mμ; band IV, 512 mμ. The ratio of absorbance at band III to that at band IV was 1.31 (rhodo type). An aqueous HCl concentration of 25% (w/v) was necessary for complete extraction of the porphyrin from ether. If allowed to stand overnight at room temperature in 25% HCl and returned to ether the porphyrin now had bands at 635 mμ, 578 mμ, 549 mμ and 508 mμ. The ratio of band III/band IV was 1.12 (rhodo type). This porphyrin was also now somewhat more soluble in aqueous HCl requiring a concentration of about 17% for extraction from ether. The chemical change brought about by the treatment in 25% HCl is not yet clear

2. This method was that of MORELL AND STEWART[18], the reaction mixture containing acetic acid, ferrous sulphate and 3% (v/v) of concentrated HCl. The iron was efficiently removed from the haematin by this method but a mixture of porphyrins was usually obtained varying in band positions and HCl number. Because of its single, unaltered porphyrin product Method 1 was preferred.

Electrophilic properties of the porphyrin substituent

In Table I it can be seen in a series of known porphyrins that the more electrophilic the substituent the further to the red does the position of band III lie. This relationship also holds for the α-bands of the pyridine haemochromes of their iron complexes and for the α-bands of their copper and zinc complexes. The porphyrin derived from milk peroxidase after treatment with HCl, has its band III in ether at 549 mμ suggesting a substitution somewhat more electrophilic than that of monoacetyldeuteroporphyrin. Its corresponding haemochrome and zinc complex band positions indicate, however, a substituent slightly less electrophilic than for that porphyrin while its copper complex has an α-band close to that of protoporphyrin. These conflicting results make it difficult to assess the electrophilic properties of the milk peroxidase porphyrin substituent. The big disparity between the copper complex and the other derivatives suggests the possibility that copper reacts with the substituent thus lowering its electron attraction.

TABLE I

Porphyrin or haemoprotein	*Porphyrin* band III in ether (mμ)*	*α-Bands (mμ)*		
		*Pyridine haemochrome** of Fe complex*	*Copper complex in ether*	*Zinc complex in ether*
Deuteroporphyrin	526	545	558	572
Protoporphyrin	537	558	570	583
Milk-peroxidase haemoprotein	—	558***–571§	—	—
Milk-peroxidase haematin	—	568§	—	—
Milk-peroxidase porphyrin	549	566§	572	589
Monoacetyldeuteroporphyrin	543	571	577	591
Monotormyldeuteroporphyrin	551	578	584	596

* These were the dimethyl esters except in the case of milk peroxidase porphyrin.
** Except for milk peroxidase derivatives the reagents were: pyridine–1 N NaOH (25:75, v/v), reducer $Na_2S_2O_4$.
*** Reagents: pyridine–0.1 N NaOH (25:75, v/v), reducer $Na_2S_2O_4$.
§ Reagents: pyridine–water (25:75, v/v), reducer $Na_2S_2O_4$.

The higher value shown in Table I for the α-band of the pyridine haemochrome formed from the haemoprotein might best indicate the electrophilic properties of the substituent. The lower value can reasonably be ascribed to alteration of the substituent by alkali.

Re-conversion of milk-peroxidase porphyrin to haematin

The porphyrin with band III at 552 mμ derived from milk peroxidase was dissolved in acetic acid and buffered with sodium carbonate or pyridine so that the spectrum was that of the neutral porphyrin. Excess ferrous sulphate was added and the solution heated to 85° for 15 min under carbon dioxide. The resultant haematin was taken into ether. The pyridine haemochrome formed from this haematin in pyridine–water (1:3, v/v) had an initial absorption band at 559 mμ shifting over 15 min to 566 mμ. This is in contrast to the ether-soluble milk peroxidase haematin which gave an immediate value of 568 mμ.

DISCUSSION

The traditional first approach to the determination of the nature of the haematin group of a haemoprotein is the study of the spectral properties of its various derivatives and determination of the position of the α-band of the pyridine haemochrome. Thus the spectra of oxidized and reduced milk peroxidase and of its compounds with carbon monoxide, cyanide, fluoride[5], and hydrogen peroxide[3,19] are closely similar in type to the corresponding compounds of horse-radish peroxidase[7] and other plant peroxidases which are known to have protohaematin as their prosthetic group. The main difference to be found is that the predominant band in the visible spectrum of each milk peroxidase derivative tends to lie somewhat towards the red compared with its corresponding derivative of horse-radish peroxidase. It is well known from study of the cytochromes that these band positions are influenced not only by the chemical nature of the haematin but also by the nature of the non pyrrolic ligands co-ordinated with the iron atom (*e.g.* the cytochromes *b*[20]). Greatest weight as to the nature of the haematin has therefore to be placed on the band positions of the pyridine haemochromes where it is assumed (although this is difficult to prove conclusively) that the haemochromes under comparison have the same (pyridine) ligands bonding at both the fifth and sixth co-ordination positions of the iron atom. In this situation it is well established from the type of data given in Table I that the more electrophilic is the conjugated substituent the further to the red does the position of its haemochrome band lie. This relationship in such an "electrophilic series" holds also for other derivatives of known compounds such as the porphyrins and their copper and zinc complexes (Table I).

The detailed study of the pyridine haemochrome of milk peroxidase reported in this paper reveals that the haematin group of this enzyme contains a substituent conjugated to the porphyrin ring considerably more electrophilic than the two vinyl groups of haematin. The evidence presented indicates that this substituent exerts an electron attraction of the same order as an acetyl group. However, the evidence given in Table I shows that here we are dealing with a haematin substituent with a number of unique properties in its lability towards alkali and certain adsorbants and in its possible reaction with some metallic ions. Some caution must therefore be observed in using the traditional methods outlined above. It would seem, in this particular case, that the true effect of this substituent can only be gauged when its structure has been established chemically.

Fundamental to the chemical study of milk-peroxidase haematin has been the search for a method of breaking the co-valent bonds between haematin and protein which does not destroy the characteristic electrophilic group. So far the only reagent found satisfactory in this respect is hydrogen bromide in acetic acid. The resultant ether-soluble haematin gives a pyridine haemochrome with properties similar to that of the intact haemoprotein, from which it is adduced that the electrophilic group is substantially unaltered.

Successful splitting of the haemoprotein by alkali indicates that the linkage to protein is probably through an ester or, possibly, an amide. An ester or amide bond linking haematin to protein would be unique. Other haemoproteins in which the haematin is co-valently bound to the protein contain thio-ether bonds (cytochrome *c*[21]; one of the bonds of Chromatium RHP-type protein[14]) or have not yet been identified

(leukocyte peroxidase; another of the bonds in Chromatium RHP-type protein).

The porphyrin obtained from the ether-soluble haematin by treatment with HBr–acetic acid–ferrous sulphate followed by standing in 25 % HCl, shows some properties of interest. The purest preparation so far obtained has a specific extinction (absorbance at 549 mμ) of only about 10.9 indicating either appreciable impurity or high molecular weight. Its purification is attended with difficulties similar to those encountered with the haematin due to lability of the electrophilic group. Of considerable interest is the fact that it is only extractable from ether by aqueous HCl when a strength of 17 % is used. This alone might indicate a basicity of the porphyrin nitrogens less than that of monoformyldeuteroporphyrin. Since the other evidence so far produced does not indicate a substituent with an electron attraction greater even than that of one acetyl group, some other factor seems likely and requires further investigation.

The significance (if any) of the formation of bile pigment-like compound as by-product to the splitting of the haematin from the apoenzyme is difficult to judge on the evidence so far available. The pigment seems still to be attached to the protein under conditions which split the haematin–protein bonds. Its insolubility in organic solvents makes its investigation unrewarding. The evidence does, however, indicate that it arises from a ferric haematin since HBr–acetic acid in the absence of $FeSO_4$ does not give a porphyrin. Furthermore, it is produced either from a haematin still co-valently bonded to the protein or, co-incident with its formation, a new bond with the protein arises.

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