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HIGH-PRESSURE LIQUID CHROMATOGRAPHY AND FIELD DESORPTION MASS SPECTROMETRY OF HEME *a*, HEME *a* DIMETHYL ESTER AND ACETYL HEME *a* DIMETHYL ESTER

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Summary

Field desorption mass spectrometry was shown to be a valid technique for determining the molecular weights of hemins and hemin esters, as well as of porphyrins. The observed base peaks of ligand-free protoheme IX, protoheme IX dimethyl ester, and protoporphyrin IX dimethyl ester correspond well to the molecular weights of these compounds and the base peak for hematoporphyrin corresponds to the molecular weight of this porphyrin minus two molecules of water. The technique was employed to confirm the molecular weights of heme *a*, heme *a* dimethyl ester, and acetyl heme *a* dimethyl ester. Heme *a* dimethyl ester was prepared by reaction of heme *a* with trimethyloxonium tetrafluoroborate and purified by high-pressure liquid chromatography. The isolated product was converted to the acetylated derivative by reaction with acetic anhydride and was subsequently purified by high-pressure liquid chromatography. A field desorption spectrum of heme *a* shows a base peak at 582 which is in agreement with previous deductions of the structure of this prosthetic group. Base peaks of the heme *a* ester and its acetylated derivative demonstrate that the two carboxyl groups have been methylated and the single hydroxyl group has been acetylated without further alteration of the molecule.

Introduction

Heme *a*, the prosthetic group of cytochrome oxidase, has been studied extensively. The structure (Fig. 1) has been deduced from chemical and physical studies and is supported by mass spectral analysis of the monomethyl ether of heme *a* dimethyl ester [1]. However, the esterification of hemes by classical procedures and their conversion to porphyrins require acidic conditions and

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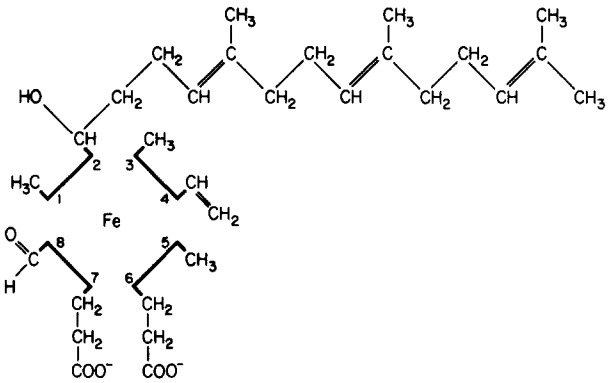

Fig. 1.

there has been some basis for suspecting modification of the prosthetic group in acid [1,2]. The involatility of free hemes and porphyrins makes characterization by electron impact or chemical ionization mass spectrometry impossible.

The present study was initiated when we realized that characterization of heme *a* might be furthered by combining three new techniques, esterification of hemes at neutrality with oxonium salts [3], purification of hemes by high-pressure liquid chromatography [4], and molecular weight determination by field desorption mass spectral analysis [4]. In this paper we report the purification and molecular weight determination of heme *a*, heme *a* dimethyl ester, and acetyl heme *a* dimethyl ester.

Materials and Methods

Reagents

Pyridine for spectrophotometry was dried over potassium hydroxide pellets and distilled from ninhydrin. Purified pyridine was stored over molecular sieves (4A, Linde) and potassium hydroxide. All other chemicals were reagent grade and were not further purified. Polyamide thin-layer chromatography plates were obtained from Gallard-Schlesinger.

Hemes and porphyrins

Hematoporphyrin dihydrochloride, protohemin IX chloride, and protoporphyrin IX dimethyl ester were obtained from Sigma. Heme *a* was isolated from bovine heart by the method of York et al. [2]. Protohemin IX dimethyl ester and heme *a* dimethyl ester were prepared using trimethyloxonium tetrafluoroborate as described by Dean et al. [3]. Heme *a* dimethyl ester was subsequently acetylated with acetic anhydride to form 2-1′-acetyl heme *a* dimethyl ester [3].

High-pressure liquid chromatography

High-pressure liquid chromatography was performed using a Waters Associates model No. M-6000 A pump fitted with an 254 nm absorbance detector (model No. 440) and a refractometer (model No. R401). Separations were

carried out on a Waters Associates μBondapak C_{18} column at a flow rate of 2 ml/min. The procedure was repeated a number of times in order to obtain enough material for further studies.

The impure sample of heme *a* dimethyl ester was applied to the column in dry chloroform and eluted with 2% water in methanol. Monitoring of the eluant for change in refractive index and absorbance at 254 nm showed three peaks. The first component to be eluted at 0.75 ml was a non-heme impurity. The second peak (at 1.45 ml elution volume) was shown by spectrophotometry and polyamide thin-layer chromatography to be a mixture of unesterified and monoestererified heme *a*. The third component, consisted of a peak at 3.63 ml with a shoulder at smaller volumes. Polyamide thin-layer chromatography and spectrophotometry of the reduced pyridine hemochrome indicate that the compound in the peak and the compound in the shoulder are indistinguishable from one another, are both homogeneous, and are indeed heme *a* dimethyl ester. Although the column was eluted with 2% water in methanol, the presence of traces of water in the chloroform used to dissolve the heme resulted in a larger proportion of the heme *a* dimethyl ester behaving as the faster migrating shoulder. Such behavior suggests a monomer-*n*mer heterogeneity, the composition of which is altered by water.

The acetyl derivative of heme *a* dimethyl ester was also purified by high-pressure liquid chromatography. The sample was dissolved in dry chloroform and a 50 μl aliquot was applied to the μBondapak C_{18} column and then eluted with absolute methanol. After non-heme containing impurities had been eluted, two partially resolved heme compounds were detected. The system was switched to the recycle mode and after three cycles the two components had been resolved. Analysis by polyamide thin-layer chromatography revealed the first component to be the heme *a* dimethyl ester and the second component to be the acetyl heme *a* dimethyl ester which was contaminated by a small amount of the unacetylated ester. Repurification of the second fraction yielded essentially a single species on polyamide thin-layer chromatography.

Pyridine hemochromes

Reduced pyridine hemochrome spectra were recorded in pyridine/water, (10 : 1, v/v). The water was included to facilitate the dissolution of the reducing agent, $Na_2S_2O_4$. The solutions were deoxygenated before the addition of the solid $Na_2S_2O_4$ by bubbling a thin stream of nitrogen (Linde, prepurified grade) through the solution in the cuvette for a few minutes. The positions and extinctions of the absorption maxima of the reduced pyridine hemochrome spectra of heme *a* were identical in pyridine/water, (10 : 1, v/v), and in absolute pyridine (absorption maxima at 429, 533, and 584 nm with relative absorbance of 4.80, 0.36, and 1.00, respectively).

Polyamide thin-layer chromatography

Polyamide thin-layer chromatography was performed using the 10% acetic acid in benzene solvent system [5]. The R_F values were somewhat concentration-dependent, higher concentrations resulting in greater R_F values. As previously reported, the R_F value decreases with increasing polarity of the deriva-

tive, viz: acetyl heme *a* dimethyl ester > heme *a* dimethyl ester > heme *a* monomethyl ester > heme *a* [3].

Field desorption mass spectrometry

Field desorption mass spectra were obtained using a Varian MAT CH5-DF mass spectrometer equipped with a field desorption/field ionization/electron impact combined ion source and PDP-11 data system. Samples of approximately 10 μg/μl were prepared as follows: protoporphyrin IX dimethyl ester in chloroform, hematoporphyrin · 2 HCl in methanol, protohemin IX chloride in dimethyl sulfoxide, protohemin IX dimethyl ester chloride in acetone, and heme *a*, heme *a* dimethyl ester, and acetylated heme *a* dimethyl ester in pyridine. The tungsten emitter wire was activated with benzonitrile. Approximately 0.5—1 μg of sample was then applied by dipping the wire into the heme solution and evaporating the solvent. Spectra were recorded at an emitter heating current of 15—24 mA. The ion source temperature was 80° C and the accelerating voltage 3 kV. The extraction plate voltage was −7 kV. The resolution of the instrument was greater than 1 part per 1000. Flash heating of the emitter wire was necessary in order to obtain spectra of heme *a* and its derivatives. The ion intensities were apparently too low to be seen over the normal scanning interval. In order to obtain the molecular ion peak of acetylated heme *a* dimethyl ester, it was necessary to scan backwards from high to low mass. Since the molecular weights of heme *a* ester and acetylated heme *a* ester were above the highest calibrated mass of the data system, the masses of these compounds were established by comparison with a standard perfluorokerosene spectrum. This reduced the accuracy of these mass determinations to ±2 mass units.

Results and Discussion

Purification of heme a dimethyl ester and its acetyl derivative by high-pressure liquid chromatography

High-pressure liquid chromatography has been shown in this study to be an excellent method for purifying heme *a* dimethyl ester and its acetylated derivative. We have employed a reverse phase method in which the compounds are partitioned between an extremely hydrophobic solid phase and a hydrophilic elutant. In this system the heme *a* derivatives are eluted in the order of their polarities, with acetyl heme *a* dimethyl ester having the greatest retention time. Heme *a* dimethyl ester was eluted with 2% water in methanol whereas the acetylated derivative was eluted with absolute methanol using the recycling mode. Both samples appeared homogeneous on polyamide thin-layer chromatography.

Whereas high-pressure liquid chromatography has been used for the isolation and quantitation of porphyrin and porphyrin esters [4,6,7], there have been no previous reports on the application of this technique to hemes and heme derivatives. Under the mild conditions employed, chemical alteration of hemes should be minimal. Thus the procedure has considerable advantage over the classical chromatographic procedures employing silicic acid and alumina which cause degradation of tetrapyrroles.

Preparation of heme a dimethyl ester and its acetyl derivative

The use of trimethyloxonium tetrafluoroborate allowed selective methylation of the two propionic acid groups of heme *a*, leaving the hydroxyl group free to be subsequently acetylated with acetic anhydride [3]. The purified heme *a* dimethyl ester gave a reduced pyridine hemochrome spectrum in pyridine/water, 10 : 1, with absorbance maxima at 585, 533, 495 (shoulder), and 430 nm with relative extinction of 1.0, 0.35, 0.46, and 4.39, respectively. The spectrum of the purified acetyl heme *a* dimethyl ester is very similar to that of the unacetylated dimethyl ester with absorbance maxima at 583, 533, 495 (shoulder), and 430 nm with relative extinctions of 1.0, 0.36, 0.40, and 4.45, respectively. As described below, field desorption mass spectrometry confirms the structural designation of both the ester and its acetylated product.

Previous attempts to prepare acetyl heme *a* dimethyl ester have been complicated by the side reactions which heme *a* undergoes during methylation and acetylation reactions. Methanolic-mineral acid not only esterifies the carboxyl groups of heme *a*, but to a lesser extent, converts its hydroxyl group to a methyl ether [1]. Reaction of heme *a* with acetic anhydride/pyridine for 12 h has been found to result in a 5 nm blue shift in the α-peak of the reduced pyridine hemochrome [8]. Since simple acetylation of the hydroxyl group would be expected to give little or no shift in absorbance maxima, the shift observed with porphyrin *a* has been attributed to cyclization of the long alkyl chain to give a substituted pyrrole- or furan-like structure [9].

Caughey et al. [10] have reported that reaction of free heme *a* with acetic anhydride in pyridine for only 10 min at 40—50° C results in the formation of a single product. The spectral change accompanying this shorter acetylation reaction was relatively small; the absorbance maxima of the reduced pyridine hemochrome shifted from 427.5, 535, 583 nm to 424, 533, 582 nm. The present study shows that acetylation of the heme *a* ester under the same conditions employed by Caughey et al. results in even less alteration in the spectrum. This finding suggests that acetylation of the side chain without subsequent isomerization reactions results in no spectral change. Surprisingly, acetylation of the same heme *a* dimethyl ester did not go to completion. Even after 25 min of incubation at 40—45° C, the reaction was only 80—90% complete as judged by polyamide thin-layer chromatography.

Field desorption mass spectrometry of model tetrapyrroles

The *m/e* values and the relative intensities of the mass peaks from the field desorption mass spectra are summarized in Table I. The spectra of protoporphyrin IX dimethyl ester show a base peak at *m/e* 590.5, which agrees very closely with its known molecular weight (590.7). The only other predominant peak in this spectrum is a peak at base peak +1. Peaks corresponding to base peak +1 and +2 are typical of field desorption mass spectra [4,11] and were also observed with the other porphyrins and hemins in this study. The observed base peak of hematoporphyrin at *m/e* 562.6 does not correspond to the molecular weight of this compound, but instead agrees very well with the molecular weight of protoporphyrin (562.7). It is apparent that a molecule of water is lost from each of the two hydroxyethyl groups during the mass spectral procedure. Such dehydration was expected since previously reported field desorp-

TABLE I

FIELD DESORPTION MASS SPECTRA OF HEMES AND PORPHYRINS

Molecular weights are those of ligand-free heme reported in the literature. Numbers in parentheses are peak intensities relative to the largest peak in that spectrum. Shown are all peaks with intensity ⩾8% of the intensity of the major peak except in the case of heme *a* dimethyl ester and the acetyl heme *a* dimethyl ester. The intensities of the major peaks in the spectra of the heme *a* derivatives are very small and only those peaks with intensities ⩾35% are presented. (a) and (b) denote two runs with aliquots of the same sample.

Compound	Molecular weight	*m/e* (% relative intensity)
Protoporphyrin IX dimethyl ester	590.7	590.5(100) 591.5(37)
Protohemin IX	616.5	616.3(100) 617.5(78) 618.4(26) 652.1(8)
Protohemin IX dimethyl ester	644.5	644.6(17) 646.5(17) 679.5(100) 680.7(23) 681.4(44) 682.6(17) 683.4(9)
Hematoporphyrin	598.7	562.6(100) 563.6(46) 564.7(13)
Heme *a*	852.9	(a) 852(100) (b) 736(12) 783(24) 787(12) 795(12) 805(24) 835(34) 834(100)
Heme *a* dimethyl ester	880.9	(a) 387(60) 688(47) 879(40) 880(35) 881(100) 882(64) (b) 858(36) 859(36) 860(42) 875(51) 876(40) 877(90) 878(100)
Acetyl heme *a* dimethyl ester	922.9	628(39) 722(39) 728(39) 857(44) 920(100) 922(58) 975(37)

tion analysis of hematoporphyrin showed dehydration to protoporphyrin [12].

The base peak of protohemin IX at *m/e* 616.3 agrees very well with its known molecular weight (616.5). This spectrum shows a smaller peak at *m/e* 652.1 which corresponds to the chloride complex of protohemin (652.0). Similarly, the spectrum of protohemin dimethyl ester shows peaks at *m/e* 644.6 and 679.5 which correspond to the molecular weight of the protohemin ester (644.5) and its chloride complex (680.0). However, in the case of the protohemin ester, the chloride complex is the larger of the two peaks. The chloride ions are present because protohemin chloride and the dimethyl ester of protohemin chloride were employed for these studies. Since the hemin, but not the porphyrins, showed this complexation with chloride, it is clear that the chloride is bound to the ferric ion. In light of such chloride binding, field desorption mass spectra of hemes must be interpreted with caution. Counter ions have been observed previously with field desorption mass spectrometry [13].

The field desorption mass spectrum of each of these tetrapyrroles was compared with an electron impact mass spectrum obtained with aliquots from the same samples using the same instrument and data system. In each case the two techniques gave essentially the same *m/e* values for the parent ion. As expected, the electron impact mass spectrum showed typical fragmentation patterns with primary cleavage at the β-carbon of the propionic side chains.

Field desorption mass spectrometry of heme a and its derivatives

The field desorption mass spectra of free heme *a* showed a peak at either *m/e* 852 or 834 using aliquots of the same sample (Table I). The peak at *m/e*

852 corresponds to the molecular weight of the structure which has been deduced for ligand-free heme *a* [10]. The peak at *m/e* 834 corresponds to the molecular weight of the structure in which a molecule of water has been lost from the α-hydroxy alkyl side chain at position 2 of the porphyrin ring.

The methylation product from the reaction of heme *a* with trimethyloxonium tetrafluoroborate shows a cluster centered at approximately *m/e* 880 which corresponds to the derivative in which the two propionic acid groups have been converted to the methyl esters. In analogy with the free heme *a*, the dimethyl ester showed a peak at base peak minus 18 which is readily explained by loss of water from the hydroxy alkyl side chain. The mass spectrum showed no peak at 894, indicating that no methylation of the hydroxy alkyl group had occurred. The acetylation product of heme *a* dimethyl ester gave an ion cluster at approximately *m/e* 920—922 (Table I) indicating that acetylation of the single hydroxyl group had been effected.

These field desorption mass spectral studies demonstrate that methylation of the carboxyl groups of heme *a* with trimethyloxonium tetrafluoroborate and subsequent acetylation of the hydroxy alkyl group with acetic anhydride proceeded without further modification of the molecule. The mass determinations of these compounds, together with that of the free heme, provide further support for the previously deduced structure of heme *a* [10]. These findings can not, of course, rule out other possible structures with the same molecular weight. Since the heme isolation, methylation, and product purification were carried out under neutral conditions, the present study provides confirmatory evidence that no acid-labile group is present on the heme *a* molecule.

Field desorption mass spectra of porphyrins, porphyrin esters, and protohemin have been reported previously [4,6,7,12]. The data presented in the present study demonstrate the applicability of this technique to hemes and heme derivatives in general. The technique is of particular value for the study of heme *a* and other hemes which are refractory to structure elucidation. Since in this technique ionization is achieved with little excess energy being transferred to the molecules, the resulting spectra are simple relative to the fragmentation patterns of electron impact mass spectra. The great advantage, then, of field desorption mass spectrometry in the study of complex or labile hemes is its ability to create a base peak which in most cases corresponds to the parent ion.

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