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Affinity Labeling of Myoglobin with Mesoheme Sulfuric Anhydride*

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ABSTRACT: Mesoheme monosulfuric anhydride reacts with apomyoglobin to form a derivative in which the heme group is covalently bound to the protein. The visible spectrum of the anhydride–myoglobin derivative is very similar to the spectrum obtained by adding mesoheme to apomyoglobin. Pepsin digestion of anhydride–myoglobin releases two major heme peptides. The amino acid compositions of these peptides suggest that the mesoheme is attached to lysine residue 45 in both cases. These results verify the site of reaction predicted from the X-ray diffraction structure of myoglobin and validate the use of this reagent as an affinity label for heme proteins.

Heme sulfuric anhydrides, in which one or both carboxyl groups of the propionic acid side chains of the heme group are activated for reaction with nucleophilic agents, have been used for the preparation of heme model compounds having specific amino acid ligands (Warme and Hager, 1970a,b). These anhydrides are also potentially useful as affinity labels for heme proteins. We have explored this possibility using myoglobins as a test case. For obvious reasons, myoglobin is the protein of choice for these preliminary studies. Myoglobin is a low molecular weight, monomeric heme protein. The sequence of amino acids in myoglobin from several mammalian species has been established and, most important, the three-dimensional crystal structure of sperm whale myoglobin has been determined. Thus, the background information is available for both predicting the reaction site of the heme anhydride with myoglobin and interpreting the final result.

In this paper we describe the purification of mesoheme monosulfuric anhydride and its reaction with apomyoglobin. Methods are outlined for the isolation of peptides containing the covalently bound heme affinity label. The results indicate that mesoheme monosulfuric anhydride reacts with lysine residue 45 in horse myoglobin to establish a covalent link. This reaction is diagrammatically outlined in Figure 1. These studies validate the use of heme anhydrides as affinity labels for heme proteins.

Experimental Section

Materials. Horse myoglobin was purchased from Pierce Chemical Co. The pepsin was a three-times-crystallized preparation obtained from Nutritional Biochemicals Corp. Eastman Chromagram sheets and triethanolamine were products of Eastman Kodak Co. All other reagents were Analytical grade.

Preparation of Apomyoglobin. Horse apomyoglobin was prepared by the method of O'Hagen and George (1960) except that the myoglobin was dissolved in 1% (w/v) sodium chloride solution and a 4.0 mM HCl–acetone solution was used. These modifications insured a more complete removal of the heme and the precipitated apoprotein was more easily soluble in water. The concentration of native apomyoglobin was determined by diluting 10 μl of apomyoglobin solution (about 10 mg/ml) to 1.0 ml with 0.1 M potassium phosphate buffer (pH 7.0) and titrating with 0.1 mM mesoheme in 0.01 M NaOH while following the increase in optical density at the Soret maximum (395 nm). The concentration of mesoheme was determined from the pyridine hemochrome

* From the Biochemistry Division, Department of Chemistry and Chemical Engineering, University of Illinois, Urbana, Illinois 61801. *Received May 7, 1970.* This is the third paper in a series dealing with heme sulfuric anhydrides. The previous paper is listed in the references (Warme and Hager, 1970b).

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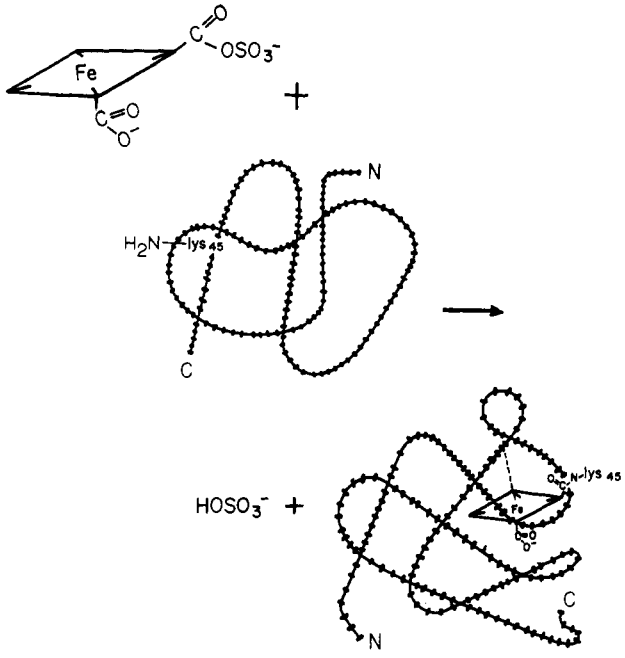

FIGURE 1: Diagrammatic representation of the reaction of mesoheme monosulfuric anhydride with apomyoglobin. Mesoheme monosulfuric anhydride is written in an abbreviated form emphasizing the carboxyl groups of the two propionic acid side chains. Myoglobin is represented in its conventional configuration, each dot represents an amino acid residue.

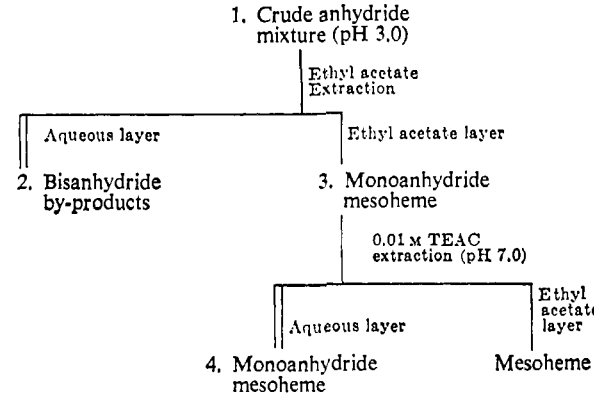

FIGURE 2: Flow diagram of mesoheme monoanhydride purification. The numbers correspond to the histidine test samples shown on the thin-layer chromatogram in Figure 3.

spectrum, using a millimolar extinction coefficient of 33.2 (Falk and Perrin, 1961).

Preparation of Mesoheme Monosulfuric Anhydride. The procedure outlined in Figure 2 was designed to isolate highly purified samples of the monosulfuric anhydride derivative. Crude mesoheme anhydride was prepared as previously described (Warme and Hager, 1970a) by dissolving 5 mg (7.5 μmoles) of the lithium chloride salt of mesoheme in 0.1 ml of 1 M SO_3–dimethylformamide solution in dimethylformamide. Triethylamine (10 μl, 73.5 μmoles) was added and anhydride formation was allowed to proceed for 30 min at room temperature (23°). The anhydride solution was then added with stirring to 1.9 ml of water at 0°, and the pH was adjusted carefully to 3.0 by addition of 1 M KOH with strong stirring to avoid local high concentrations of base which would hydrolyze the anhydride. The solution was then extracted six times with 3-ml portions of cold ethyl acetate (0°). At each extraction step, the mixture was shaken vigorously for 1 min on a Vortex mixer and then centrifuged briefly to separate the layers. The ethyl acetate extracts were combined and washed twice as above with 3-ml portions of water, and then back-extracted five times with 3-ml portions of TEAC[1] buffer (pH 7.0). During this extraction with the amine buffer, the anhydride formed an emulsion at the interface and was not extracted into the aqueous phase until the second or third extraction step. The darkest colored aqueous phases (usually the third and fourth extracts) were pooled and this fraction is herein called the pH 7 extract. During the entire extraction procedure above, the temperature was kept below 10°. The subsequent reaction of the monoanhydride with apomyoglobin was carried out within 1 hr in order to avoid hydrolysis of the heme anhydride.

Reaction of Mesoheme Monoanhydride with Apomyoglobin. The amount of anhydride solution required for reaction with apomyoglobin was determined by spectrophotometric titration with the pH 7 extract, using the changes in absorbance at 395 nm to determine the end point. The concentration of anhydride was determined from the pyridine hemochrome spectrum. A 10-mg/ml apomyoglobin solution (2.5 ml) was diluted with ten volumes of 0.1 M TEAC buffer (pH 7.0) and 1.0–1.1 equiv of pH 7 extract (6 ml) was added with stirring. One volume (2.5 ml) of 2.0 M Tris was added to raise the pH to 8.2 and then the reaction mixture was stirred for 1 hr at room temperature (23°). The solution became turbid after addition of the pH 7 extract, but the amount of precipitate did not noticeably increase during the reaction. After 1 hr, the pH was adjusted to 7.0 with 1 M HCl and the suspension was dialyzed for at least 10 hr against 40 l. of deionized water. After adding 2.0 ml of 2% (w/v) sodium chloride solution, the suspension was lyophilized to dryness, dissolved in 4.0 ml of distilled water, and centrifuged to remove the red precipitate. The supernatant was again subjected to the acid–acetone treatment described above in order to remove the unreacted heme.

Pepsin Digestion of Anhydride–Myoglobin. Glacial acetic acid (0.15 ml) was added to the dialyzed, acid–acetone-treated anhydride–myoglobin solution (about 4 ml) and the pH was adjusted to 2.0 with 4 N HCl. Pepsin (1.0 mg) was added and the digestion was allowed to proceed for 24 hr at 38°. In some cases, another 1 mg of pepsin was added at this point and digestion was continued for 24 hr to complete the hydrolysis. The hydrolysate was lyophilized to dryness, dissolved in 0.15 ml of 10% (v/v) acetic acid solution, and then centrifuged to remove a brownish-white precipitate. The precipitate was washed with 0.05 ml of 10% acetic acid to remove most of the residual heme peptides, and the supernatant was combined with the main solution.

Purification of the Peptic Heme Peptides. The concentrated pepsin digest was spotted on a 7-in. line on Whatman No. 3MM paper slightly moistened with pH 3.7 buffer (10 ml of acetic acid and 1 ml of pyridine in 289 ml of water). Elec-

[1] Abbreviation used is: TEAC, 0.01 M triethanol–ammonium chloride.

trophoresis was run at 0° for 4 hr at 56 V/cm on a Pherograph high-voltage electrophoresis apparatus. After drying, the brown heme area at the origin was eluted with 10% (v/v) pyridine solution in a descending chromatography chamber. The eluate (about 25 ml) was evaporated *in vacuo*, dissolved in 0.2 ml of 10% pyridine, and electrophoresed for 2 hr as above but using pH 6.4 buffer (100 ml of pyridine and 4 ml of acetic acid in 900 ml of water). After drying the electrophoretegram, the heme-containing bands were eluted with 10% pyridine and evaporated to dryness. The concentrations of the heme peptides were determined from the spectrum of the 10% pyridine eluates using an extinction coefficient of 10.3 at 520 nm. The evaporated eluates from the electrophoretogram were redissolved in 0.1–0.2 ml of 50% (v/v) pyridine. Further purification was achieved by ascending thin-layer chromatography (14–15 cm) on Eastman Chromagram silicic acid sheets (10 mμmoles/cm) using butanol–pyridine–acetic acid–water (15:10:3:12, v/v) for the development. After drying in a stream of warm air, the heme-containing areas on the thin-layer plates were scraped and the heme peptides were eluted with three 0.3-ml portions of 50% pyridine on a small sintered-glass funnel.

Amino Acid Analyses. The purified heme peptide fractions (5–15 nmoles) were hydrolyzed in 1 ml of 6 N HCl for 24 hr at 110° in evacuated, sealed hydrolysis vials. The hydrolysates were evaporated to dryness *in vacuo*, dissolved in buffer, and centrifuged to remove the brownish precipitate which results from degradation of the organic binder during elution of the heme peptides from the Eastman Chromagram silicic acid sheets. A Beckman Model 120C amino acid analyzer was used for the analyses.

Results

Isolation of Mesoheme Monosulfuric Anhydride. The ethyl acetate extraction of mesoheme monoanhydride from the crude anhydride mixture was performed at pH 3, where mesoheme has a net positive charge on the heme iron, the monoanhydride is neutral, and the bisanhydride has a negative charge, thus favoring selective extraction of the monoanhydride. TEAC was chosen for back-extraction of the heme anhydride into the aqueous phase because the organic nature of this buffer allows it to partition between the organic and aqueous phases. This is suggested by the observation that neither potassium acetate nor potassium phosphate buffers were effective for extraction of the anhydride from ethyl acetate.

Samples were removed at various stages during the preparation of the monoanhydride and analyzed by the histidine assay (Warme and Hager, 1970a) for mono- and bisanhydride. Thin-layer chromatography gave the results shown in Figure 3. Sample 1 was taken from the aqueous solution after adjusting to pH 3, and shows the presence of mono- and bisanhydrides in about equal amounts, as well as a small amount of mesoheme. The aqueous layer after extraction six times with ethyl acetate (sample 2) also contains both mono- and bisanhydride as well as traces of impurities with low R_F's. Sample 3 was removed from the combined ethyl acetate extracts and sample 4 was taken from the pH 7 extract. Both indicate the presence of monoanhydride along with smaller amounts of mesoheme. Judging from the relative quantities of the samples spotted on this plate, it can be said that most of the bisanhydride is hydrolyzed to monoanhydride at the low pH of the aqueous phase during the ethyl acetate extraction. Thus, performing the ethyl acetate extraction at pH 3 not only optimizes conditions for selective extraction of the monoanhydride, but increases the yield of the monoanhydride.

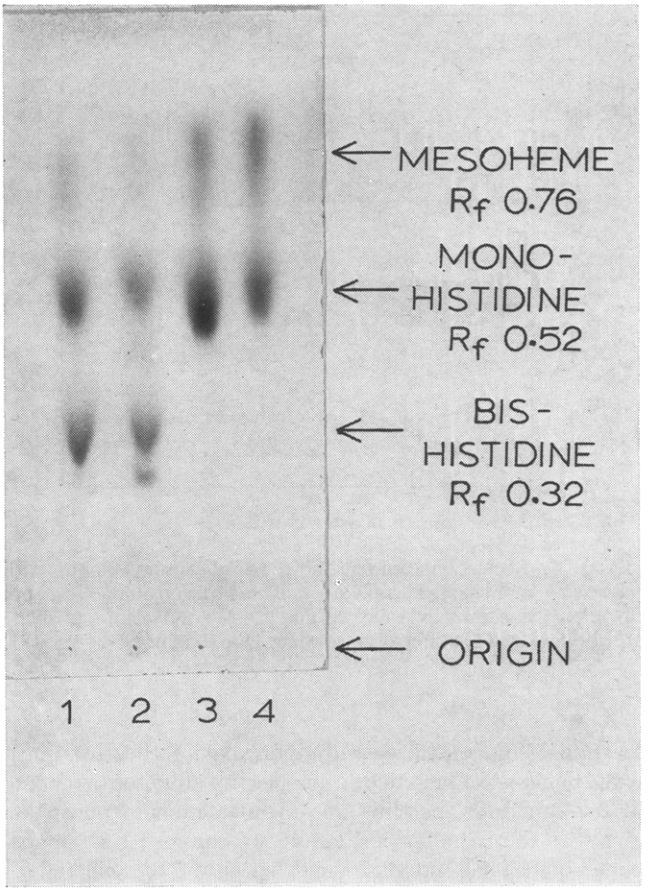

FIGURE 3: Thin-layer chromatography on samples from purification of mesoheme monoanhydride. Samples were removed at the following stages and reacted with histidine: (1) crude anhydride mixture at pH 3, (2) aqueous residue, (3) ethyl acetate extract, and (4) TEAC buffer extract (pH 7.0).

Pyridine hemochrome analysis on the pH 7 extract gave nearly the same extinction coefficient ratios as mesoheme, in contrast to previous results with the crude anhydride mixture (Warme and Hager, 1970a). However, the pyridine hemochrome spectrum of the aqueous residue after extraction with ethyl acetate had abnormal ratios, thus indicating the presence of some impurities of undefined structure. Thus, the extraction procedure also removes some undesirable impurities which are present in the crude anhydride mixture.

Preparation and Properties of Anhydride–Myoglobin. Apomyoglobin has a high affinity for mesoheme monoanhydride, as shown by the lack of curvature in the titration curve in Figure 4. However, comparison with the titration curve using mesoheme (Figure 5), which was performed on an identical aliquot of apomyoglobin, indicates that only about 46% of the purified anhydride heme is capable of recombination with apomyoglobin. TEAC buffer was used during

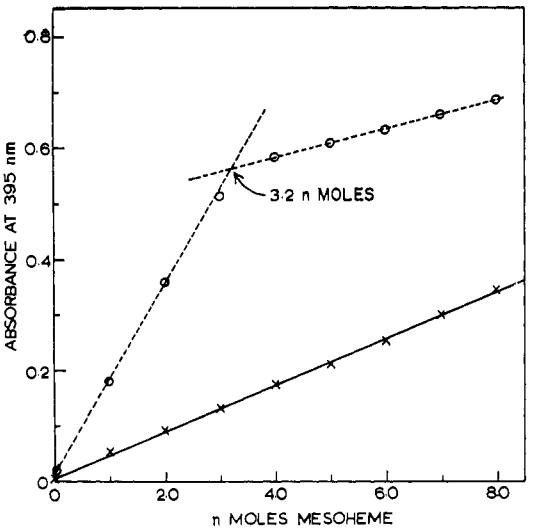

FIGURE 4: Spectrophotometric titration of apomyoglobin with mesoheme monosulfuric anhydride. The titration curve for apomyoglobin is shown as a dashed line (O- - - - - O). Aliquots of the anhydride added to buffer alone produced the solid line (X———X).

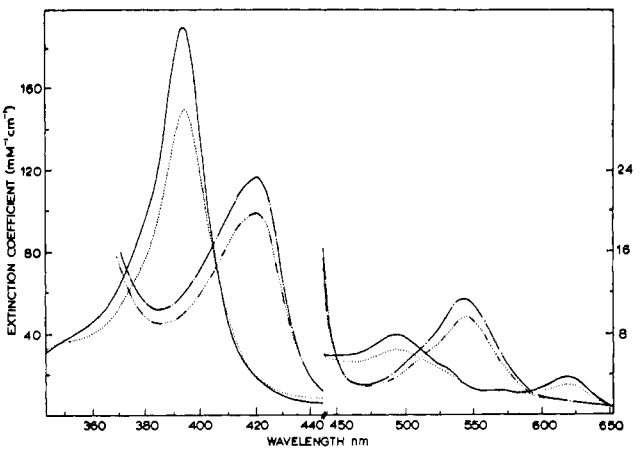

FIGURE 6: Spectral comparison of anhydride myoglobin and mesoheme myoglobin. (———) Mesoheme myoglobin oxidized, (·———·) reduced, (. . . . .) anhydride–myoglobin oxidized, and (—·—·—) reduced. Concentrations were 5.0×10^{-6} and 2.5×10^{-5} M in 0.1 M phosphate buffer (pH 7.0) for the Soret and α–β regions, respectively. The extinction coefficients are somewhat low, as mentioned in the text.

reaction of the anhydride with apomyoglobin because phosphate buffer was found to be a competitive inhibitor to binding of the anhydride, possibly due to interaction of phosphate ions with the lysine at position 45. In contrast, 0.1 M phosphate buffer had no observable effect on the binding of mesoheme. Tris (free base) was used to initiate the reaction of the anhydride with the protein so that any anhydride molecules which were not bound to the protein would react with the amino group of Tris instead of reacting nonspecifically with lysines or N-terminal amino groups on the surface of the protein.

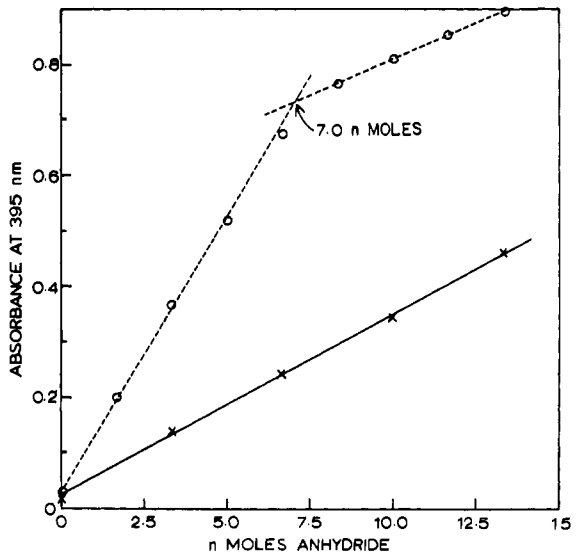

FIGURE 5: Spectrophotometric titration of apomyoglobin with mesoheme. The titration curve for apomyoglobin is shown as a dashed line (O- - - - - O). Aliquots of mesoheme added to buffer alone produced the solid line (X———X).

The spectrum of anhydride–myoglobin after acid–acetone treatment is almost identical with the spectrum of mesoheme myoglobin but has generally lower extinction coefficients, as shown in Figure 6. The concentrations of mesoheme and anhydride–myoglobin were determined by pyridine hemochrome analyses. Dilution of the dialyzed anhydride–myoglobin solution into 0.1 M potassium phosphate buffer (pH 7.0) gave a turbid solution, so the solution was centrifuged for 5 min at 20,000g before making the spectral measurements. A small red pellet was separated from the more concentrated sample used for the spectrum between 450 and 650 nm so the extinction coefficients of the anhydride myoglobin in this region are undoubtedly slightly low. The mesoheme myoglobin sample was prepared by adding a known amount of mesoheme to an excess of apomyoglobin.

Purification of the Myoglobin Heme Peptides. The results of two experiments on anhydride–myoglobin will be presented. The results were essentially the same for these two experiments, but the amino acid compositions of some of the heme peptide fractions showed significant amounts of impurities. In these cases, analyses of the corresponding fractions from both experiments will be given.

As mentioned in Methods, about one-third of the anhydride–myoglobin could be removed as a precipitate after dialysis and concentration of the reaction mixture. In expt 1, the soluble and insoluble anhydride–myoglobin fractions were carried separately through the pepsin digestion and purification of the heme peptides whereas in expt 2, these fractions were combined before purification. The yield of soluble anhydride–myoglobin was 44% in expt 1 and 67% in expt 2 based on the amount of recombinable apomyoglobin used.

Thin-layer chromatography in butanol–pyridine–acetic acid–water (15:10:3:12, v/v) on the pepsin digests of the soluble anhydride–myoglobin fraction from expt 2 showed a clear increase of the high R_F heme peptide I (see below) at the expense of the low R_F heme peptide III when the

amount of pepsin and the time of digestion was doubled. Thin-layer chromatography on the pepsin digests of the insoluble anhydride–myoglobin fraction from this experiment showed a significant amount of unreacted heme which was not removed even by 0.12 M HCl–acetone treatment at room temperature. The heme peptide spots were much less clearly defined than the spots in the soluble fraction, which suggested that the insoluble fraction digest also contained some heme peptides resulting from nonspecific reaction of mesoheme anhydride with denatured myoglobin.

The electrophoresis step at pH 3.7 is quite effective for removing neutral and basic nonheme peptides from the pepsin digest. These contaminants migrate to the cathode while the heme peptides remain within 2 in. from the origin. Electrophoresis at pH 6.4 removes the acidic nonheme peptides and resolves the heme peptides into several bands. One sharp band migrated about 1 in. from the origin on the anodic site (fraction I), another remained at the origin (fraction II), and a third diffuse band spread from the origin to 2 in. beyond it on the anodic site. This third band overlapped a band of neutral nonheme peptides detected by spraying a side strip with ninhydrin, so this band was eluted as two fractions, called IIIA and IIIB. Fraction IIIA ranged from 0.2 to 1.0 in. from the origin and was contaminated with nonheme peptides, while fraction IIIB ranged from 1 to 2 in. from the origin and was essentially free of nonheme peptides. The major heme peptide component in each of these fractions was still contaminated with traces of nonheme peptides and other heme peptides, so these impurities were removed by the final thin-layer chromatography step. Fraction I contained two major heme bands with R_F 0.67–0.74 (I-1) and 0.80–0.86 (I-2). Fraction II had a heme peptide band at R_F 0.65–0.71 (II-1) and another diffuse band at R_F 0.74–0.86 (II-2). The nonheme peptides in fraction IIIA were eliminated by preliminary thin-layer chromatography in butanol–acetic acid–water (4:1:1, v/v). In this solvent, the heme peptides had R_F 0 to 0.1, while the nonheme peptides had much higher R_F's. The low R_F area was scraped from the plate, eluted with 50% pyridine, evaporated to dryness, redissolved in 0.1 ml of 50% pyridine, and then thin-layer chromatography was carried out in butanol–pyridine–acetic acid–water as described above. The major heme peptide moved in a diffuse zone with R_F 0.47–0.56 (IIIA-1) and was preceded by a lighter band at R_F 0.58–0.70 (IIIA-2). Fraction IIIB gave a diffuse zone similar to IIIA-1, but there appeared to be a slight separation between the low and high R_F regions, so the R_F 0.50–0.56 (IIIB-1) and R_F 0.56–0.63 (IIIB-2) regions were eluted separately.

A summary of the purification steps used in both experiments is given in Table I along with the yields at the various stages for expt 2. The proportions of the various heme peptide fractions were similar in expt 1, although the total yield of heme peptides after thin-layer chromatography was only 8.4% based on the amount of myoglobin used. This purification procedure was designed to allow isolation of all of the heme peptide products rather than to selectively purify the major products. The concentrations of the various heme peptide fractions were monitored by thin-layer chromatography at each stage of the purification to guard against disproportionate losses of some of the minor components. The final yields of the purified heme peptides were found to be roughly proportional to the amounts in the pepsin digest. The degree of nonheme peptide contamination at each stage was also determined by spraying the same plate with ninhydrin. Except for fraction IIIA, the heme peptides were virtually free of nonheme peptide contamination after electrophoresis at pH 6.4. Although the final yields of some of the purified heme peptides were low, all were isolated in sufficient quantity for amino acid analysis on the high-sensitivity amino acid analyzer. However, working with low concentrations of peptides also increases contamination problems. This is evident from the amino acid compositions given in Table II. In this table, trace contaminants are designated by plus signs as explained in the footnote. Serine, glycine, and alanine contamination was particularly troublesome in many cases. The concentrations of the heme peptide solutions were determined from their spectra in 50% pyridine, using an extinction coefficient of 10.3 at 520 nm. The relative yields of the various fractions are indicated at the bottom of the columns and some amino acid compositions of peptides from horse myoglobin are included in Table II for comparison.

TABLE I: Summary of Purification and Yields of Anhydride Myoglobin Heme Peptides (Expt 2).

Sample		mμmoles	Percentage of Previous Step	Cumulative Percentage
Myoglobin		1162		100
Apomyoglobin		803	69	69
Anhydride–myoglobin (soluble)		535	67	46
pH 3.7 electrophoresis (soluble and insoluble)		615		53
pH 6.4 electrophoresis	I	220.0	35.8	18.9
	II	92.5	15.0	8.0
	III	93.5	15.2	8.0
	IV	91.0	14.8	7.8
	Sum	497.0	80.8	42.7
Thin-layer chromatography	I-1	135.2	77.4	14.6
	I-2	34.8		
	II-1	17.4	42.4	3.4
	II-2	21.8		
	IIIA-1	11.6	19.6	1.6
	IIIA-2	6.7		
	IIIB-1	22.0	48.4	3.8
	IIIB-2	22.0		
	Sum	277.5		23.4

Discussion

Mesoheme sulfuric anhydride has several properties which commend it for reaction with proteins. The sulfuric anhydride group has a high-potential reactivity with ε-amino groups of proteins, thus allowing reaction under mild conditions (pH 8–9). Mesoheme anhydride is much more soluble in

TABLE II: Amino Acid Compositions of Anhydride–Myoglobin Heme Peptides.

	Residues/Heme[a]													
	Group 1				Group 2			Group 3						
Expt:	1	2	Mb	2	1	2	2	1	1	1	Mb	2	2	2
Fraction:	I-1	I-1	43–45	II-1	I-2	I-2	II-2	IIIA	IIIB-1	IIIB-2	41–51	IIIB-1	IIIB-2	IIIA-1
Lysine	0.96	0.95	1.00	1.06	0.42	0.50	0.91	4.33	4.30	4.26	4.00	3.68	3.38	3.52
Histidine				0.27		+	0.31	0.96	1.06	0.98	1.00	1.05	0.73	1.04
Arginine														++
Aspartic acid	0.99	0.90	1.00	0.62	0.31	++	0.29	1.09	1.09	0.94	1.00	0.98	0.78	1.21
Threonine	+			++	+	+	++	1.02	0.87	0.82	1.00	0.68	0.89	0.75
Serine	+	++		++	+	0.39	0.34	++	++	++		1.12	0.26	0.89
Glutamic acid	++	++		0.34	+	++	0.44	1.37	1.13	0.83	1.00	1.15	0.58	2.08
Proline										++				
Glycine	++	++		0.38	+	0.62	0.59	0.41	0.41	0.39		0.94	0.43	1.60
Alanine	++	+		0.29	++	++	0.32	0.62	0.43	++		0.71	++	1.04
Valine	+			+	+		0.25		++	++				0.31
Isoleucine	+			++	++			+	++	++		++		0.26
Leucine	+			0.27	0.29	0.43		1.21	1.13	1.32	1.00	0.91	0.91	1.13
Tyrosine					+				++					+
Phenylalanine	1.14	0.87	1.00	0.57	0.35	0.47		2.17	2.38	2.25	2.00	1.04	1.16	1.12
Yield	(45.8)	49.8		6.4	(24.4)	12.8	8.0	(7.8)	(5.9)	(5.9)		8.1	8.1	4.3

[a] + indicates 0.05–0.15 residue/heme, ++ indicates 0.15–0.25 residue/heme.

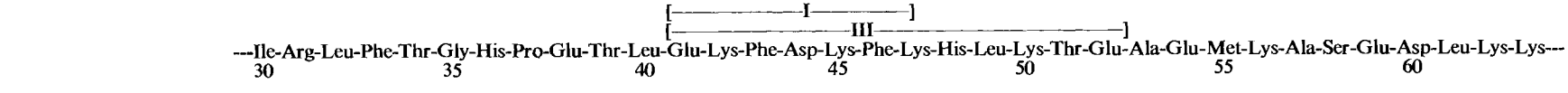

FIGURE 7: Partial sequence of horse myoglobin. The major heme peptides isolated from the peptic digest of anhydride myoglobin are derived from the indicated areas of the sequence. This partial squence was taken from the complete sequence determined by Boulanger *et al.* (personal communication).

water than mesoheme, and hydrolyzes slowly under the mild conditions required for reaction with the protein. These properties are essential for reaction with proteins in their native state, since nonaqueous solvents and high pH conditions tend to denature proteins, especially apoproteins which are already somewhat destabilized for lack of the heme group. Furthermore, the small size of the sulfuric anhydride group in comparison to other possible activating groups should minimize steric hindrance to the binding of the activated derivative in the heme crevice. In several heme proteins, the propionic acid side chains have been implicated in the binding of heme to the protein, probably due to interaction with ϵ-amino groups of lysine (Maehly, 1961; Schicha *et al.*, 1963). The fact that the sulfuric anhydride also has a negative charge should allow the same interactions to occur with mesoheme anhydride. Finally, the electrostatic attraction of the heme anhydride for amino groups in the protein should facilitate reaction. Another factor which undoubtedly affects the reaction with heme proteins is that in cases where the protein normally interacts with the heme through lysine, there is a very high effective concentration of that lysine near the site of reaction. Some combination of these factors probably accounts for the rapid reaction of the anhydride with apomyoglobin at pH 8, which is far below the normal pK of lysine.

The reasons for using mesoheme rather than protoheme for these experiments were given previously (Warme and Hager, 1970a). Due to the asymmetric disposition of the propionic acid side chains in mesoheme IX, two isomers of the monosulfuric anhydride are possible. In some heme proteins, notably cytochrome b_{562}, both isomers may react with the protein at different sites (Warme and Hager, 1970c). If a mixture of mono- and bisanhydrides is reacted with the protein in such cases, a complex mixture of derivatives can be expected, including ones resulting from reaction of each isomer of the monoanhydride and another in which both propionate groups are covalently attached to the protein. The latter product may be difficult to digest with enzymes and the resulting double heme peptides may also be more difficult to purify. Thus, it is generally advisable to use the monoanhydride derivative for reaction with heme proteins, although the bisanhydride may produce some interesting derivatives in certain cases.

The fact that only about 46% of the mesoheme monoanhydride preparation is capable of recombination with apomyoglobin suggests preferential binding of one of the two isomers of the monoanhydride. However, the fact that similar titration results were subsequently obtained for cytochrome b_{562} would point to another interpretation (Warme and Hager, 1970c). No impurities other than mesoheme were detected by reaction with histidine or pyridine hemochrome analyses on the pH 7 extract. The highest yield of anhydride–myoglobin ever obtained was 67% in expt 2 cited earlier, in contrast to yields approaching 100% for reaction with cytochrome b_{562} under similar conditions. In some early experiments with myoglobin, the crude anhydride mixture was used. About 70% recombination was achieved in this case, and yields of anhydride–myoglobin were in the range of 35–45%, as expected for reaction of only one isomer. One possible interpretation of these results is that some stable aggregate of the anhydride is present in the pH 7 extract which does not readily recombine with apomyoglobin. However, under the altered conditions for reaction (pH 8.2), more of the reactive isomer may be released. This would account for the 67% yield of anhydride–myoglobin in expt 2 without contradicting the hypothesis that only one isomer of the monoanhydride reacts with apomyoglobin.

The amino acid sequence of horse myoglobin around Lys_{45} is shown in Figure 7. Peptide I-1, which was isolated in highest amount in both experiments, was unambiguously identified with residues 43–45 in horse myoglobin. This peptide has the sequence Phe-Asp-Lys, so in this case it is clear that the mesoheme anhydride has reacted with the ϵ-amino group of lysine-45. The fractions in group 2 contain predominantly free heme, since less than one residue per heme of each amino acid was found. The presence of small amounts of amino acids in these fractions may indicate contamination with a wide variety of small heme peptides which could result from nonspecific reaction with lysine groups on the protein. However, in this case, at least one lysine should occur in each heme peptide, so nonheme peptide contamination would seem more likely.

The heme peptides in group 3 all have compositions suggesting that they are derived from residues 41 to 51 in myoglobin. Residues 41 and 52 are both glutamic acid. In general, cleavage by pepsin at glutamic acid is unfavorable, although the glutamic acid at position 17 in the A chain of insulin is a major point of pepsin cleavage (Bovey and Yanari, 1960). The neighboring sequence 43 to 52 has the same composition except for one less lysine. This would require cleavage at Phe_{43} and Ala_{53}, which would appear to be more favorable cleavage points. However, all three of the isolated heme peptides in expt 1 gave at least four lysine residues per heme, so the former sequence is probably correct. Because of the high lysine content of this region in myoglobin, the point of anhydride attachment is unclear. Thin-layer chromatography on samples removed from the pepsin digest at various times showed an increase in the high R_F heme peptide (I) at the expense of the lower R_F heme peptide (III). Furthermore, the high pepsin concentration (5%) and long digestion time (48 hr) indicate that the cleavages at Phe_{43} and/or Phe_{46} are quite slow. This would suggest attachment of the heme to Lys_{45}, where the bulkiness of the heme group would sterically hinder cleavage by pepsin.

The greater purity of the heme peptides from expt 1 doubtless reflects the fact that only the soluble portion of the anhydride–myoglobin was used in this experiment. The insoluble fraction in this case was also digested with pepsin and the heme peptides were purified in the usual manner. The relative yields of the identifiable heme peptide fractions were 15% (I-1), 19% (I-2), and 3% (III), but even these fractions were badly contaminated with extraneous amino acids. The amino acid compositions of the remaining fractions (67%) indicated a very heterogeneous mixture of peptides. Thus, the insoluble anhydride–myoglobin fraction (about one-third of the product of expt 1) consists largely of unreacted heme and a variety of products which probably result from nonspecific reaction of the anhydride with denatured apoprotein. This probably accounts for the impurity of the group 3 heme peptide fractions from expt 2, since the pepsin digests of the soluble and insoluble fractions were combined in this experiment.

In summary, about 56% of the purified heme peptides have approximately the composition of residues 43–45

(I-1, II-1), and 20% have approximately the same composition as residues 41–51 (IIIB-1, IIIB-2, and IIIA-1). About 21% appears to be unreacted heme, which leaves only 3% unaccounted for in expt 2. In this experiment, the yield of purified heme peptides was 34% based on the amount of native apomyoglobin used for the reaction. Thus, it seems reasonable to conclude that the covalent attachment of the anhydride is at Lys_{45} in all cases.

Position 45 in sperm whale myoglobin is an arginine residue and the X-ray diffraction structure shows that this arginine interacts with one of the propionic side chains of the heme (Kendrew, 1961). However, the sequences of myoglobin from all other species examined so far contain a lysine at position 45, so presumably this lysine replaces arginine as the residue that interacts with the propionate side chain. The fact that the spectrum of anhydride–myoglobin is so similar to that of mesoheme–myoglobin supports this conclusion, since any strain on the heme ring resulting from reaction at a lysine further from the heme should alter the spectrum by disruption of the normal ligand structure.

The reaction of mesoheme sulfuric anhydride with myoglobin establishes that mesoheme sulfuric anhydride reacts with groups near the normal heme binding site of the apoprotein. This method should be applicable to heme proteins in general and can be used as a tool to study the protein structure in the immediate environment of the heme.

References

Bovey, F. A., and Yanari, S. S. (1960), *Enzymes 4*, 63.
Falk, J. E., and Perrin, D. D. (1961), *in* Haematin Enzymes, Falk, J. E., Lemberg, R., and Morton, R. K., Ed., London, Pergamon.
Kendrew, J. C. (1961), *Sci. Amer. 205* (6), 96.
Maehly, A. C. (1961), *Nature 192*, 630.
O'Hagan, J. E., and George, P. (1960), *Biochem. J. 74*, 424.
Schicha, H., Hackett, D. P., and Funatsu, G. (1963), *J. Biol. Chem. 238*, 1156.
Warme, P., and Hager, L. P. (1970a), *Biochemistry 9*, 1599.
Warme, P., and Hager, L. P. (1970b), *Biochemistry 9*, 1606.
Warme, P., and Hager, L. P. (1970c), *Biochemistry 9*, 4244.

Mesoheme Sulfuric Anhydride as a Heme Protein Structure Probe. Reaction with Cytochrome b_{562}*

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ABSTRACT: Mesoheme monosulfuric anhydride reacts at three distinct sites in cytochrome b_{562} from *Escherichia coli*. The heme peptides resulting from tryptic hydrolysis of anhydride-b_{562} were purified and their amino acid compositions were correlated with the known amino acid sequence of cytochrome b_{562}. The sites of reaction are consistent with the hypothesis that the three-dimensional structure of cytochrome b_{562} is similar to that of myoglobin as was previously suggested on the basis of amino acid sequence homology.

The experiments described previously on the reaction of mesoheme monosulfuric anhydride with myoglobin (Warme and Hager, 1970b) pave the way for reaction of this reagent with heme proteins having unknown tertiary structures. Since the anhydride reacts quite specifically with lysine-45 of myoglobin, which is in a favorable position to interact electrostatically with one of the heme propionic acid groups, it is reasonable to expect a relatively specific reaction with such groups in some other heme proteins.

This paper describes the reaction of mesoheme monoanhydride with apocytochrome b_{562}. This cytochrome was first purified by Itagaki and Hager (1966). Several properties of this protein commend it for studies of this type. In contrast to many other *b*-type cytochromes which are bound to membranes, b_{562} can be extracted with the soluble fraction and is relatively easy to purify. Cytochrome b_{562} is one of the smallest of the *b*-type cytochromes (110 amino acid residues) and the complete sequence has been determined by Itagaki and Hager (1968). Thus, the locations and sequences of the heme peptides can be deduced directly from their amino acid compositions. Furthermore, cytochrome b_{562} contains only two histidines and the current hypothesis is that both are ligands to the heme. Thus, the sites of covalent attachment of the heme can be related to the positions of these proposed ligands. As a further stimulus for these studies, some sequence homology between myoglobin and cytochrome b_{562} has been noted, and the reaction with mesoheme monoanhydride provides a means of testing the hy-

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