(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.

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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-

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

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pothesis that the tertiary structures of these proteins are similar.

Experimental Section

Purification of Cytochrome b_{562} and Preparation of the Apoprotein. Cytochrome b_{562} was purified by the method of Itagaki and Hager (1966). The preparation used in these studies had been twice crystallized and showed a ratio of absorbancy at 562 nm (α band, reduced) to 280 nm (oxidized) of 1.69. The apoprotein was prepared (method I) as described by Itagaki and Hager (1966). The average yield of native apoprotein was about 90%. The apoprotein solution could be stored at −20° and was not noticeably damaged by freezing and thawing. The concentration of native apo-b_{562} was determined by spectrophotometric titration with protoheme or mesoheme, measuring the increases of the absorbancy at 418 or 406 nm, respectively. The heme solutions (0.1 mM) were freshly prepared in 5 mM NaOH and standardized by pyridine hemochrome analyses in 2.1 M pyridine–0.075 M NaOH, reduced anaerobically with $Na_2S_2O_4$ according to the methods of Paul *et al.* (1953). α-band extinction coefficients of 33.2 and 34.4 were used for the pyridine hemochromes of mesoheme and protoheme, respectively (Falk and Perrin, 1961).

Reaction of Mesoheme Monosulfuric Anhydride with Apo-b_{562}. The monoanhydride derivative of mesoheme was prepared by the extraction technique described in the previous paper (Warme and Hager, 1970c). The amount of monoanhydride required for complete reconstitution of the holoprotein was determined by spectrophotometric titration of the apoprotein, measuring the absorbancy changes at 406 nm. The apoprotein prepared from 10 mg of b_{562} was dissolved in 20 ml of 0.1 M potassium phosphate buffer (pH 7.0) and diluted with 220 ml of distilled water. Mesoheme monoanhydride (1 equiv) was added and after allowing several minutes for noncovalent binding of the heme to the apoprotein, the pH was raised to 8.6 by addition of 20 ml of 2 M Tris (free base). After 15 min at room temperature, the pH of the reaction mixture was adjusted to 7.0 with 1 N HCl. At this point, the solution contained predominantly anhydride-b_{562}, in which the mesoheme is covalently bound. The solution was lyophilized to dryness and the residue was dissolved in 8 ml of distilled water and dialyzed overnight against 4 l. of 4 mM potassium phosphate buffer (pH 7.0). The dialysate was again concentrated by lyophilization and the residue was dissolved in 2 ml of distilled water. For the spectral studies on anhydride-b_{562}, any noncovalently bound heme was removed by acid–acetone treatment in the manner used for preparing apo-b_{562}. In some cases, the anhydride-b_{562} was denatured as described below without preliminary removal of noncovalently bound heme, since the denaturation treatment accomplished the same purpose.

Denaturation and Trypsin Digestion of Anhydride-b_{562}. The anhydride-b_{562} preparation was denatured by injecting the concentrated solution (see above) in small portions into 80 ml of 0.12 M HCl–acetone (1 ml of 12 N HCl in 100 ml of acetone) with strong stirring (method II). After standing for 10 min at room temperature, the brown precipitate was collected by centrifugation, dried *in vacuo*, and dissolved in 0.8 ml of distilled water. The solution was then mixed with 40 μl of 0.1 M potassium phosphate buffer (pH 7.0) and the pH was adjusted to 7.2 with potassium hydroxide. The digestion was initiated by addition of 40 μl of 6.25 mg/ml of PTCK[1]-treated trypsin (Calbiochem) in 1 mM HCl (2.5% w/w). The pH was held between 6.8 and 7.2 during the digestion (10–20 hr at 37°). The digest was then lyophilized to dryness.

Purification of the Tryptic Heme Peptides. The lyophilized residue was dissolved in 0.15 ml of 10% (v/v) acetic acid and centrifuged to remove a brownish-white precipitate. The precipitate was washed with 0.05 ml of 10% acetic acid to extract most of the residual heme peptides. The heme peptides were purified by the methods previously described for purification of the peptic heme peptides of myoglobin (Warme and Hager, 1970c). The combined supernatants were electrophoresed at pH 3.7 (4 hr), the heme peptide band was eluted with 10% pyridine and the eluate was concentrated. The heme peptides were then electrophoresed at pH 6.4 (3 hr) and the major heme bands were eluted, concentrated, and purified by thin-layer chromatography using the methods described for the heme peptides from anhydride–myoglobin (Warme and Hager, 1970c).

Pronase and Chymotrypsin Digestion of the Tryptic Heme Peptides. About 30-nmole samples of purified heme peptide fractions II and IV (described under Results) were dissolved in 0.1 ml of 0.05 M potassium phosphate buffer (pH 7.0) and 0.1 ml of 0.05 M Tris-chloride buffer (pH 8.0), respectively. Heme peptide IV was quite insoluble, so 10 μl of 0.3 M NaOH was added and the solution was heated to 70° to solubilize the peptide. The solution was then readjusted to pH 8. Pronase (0.01 mg) was added to fraction II and α-chymotrypsin (0.01 mg) was added to fraction IV. Both digestions were carried out at 38°. After 4.5 hr, another portion of enzyme (total ~50% (w/w)) was added to each and the digestion was continued for an additional 12 hr at 38°. The digests were purified by thin-layer chromatography in butanol–pyridine–acetic acid–water (15:10:3:12, v/v). The major product from the pronase digest (II + P) had an R_F of 0.59–0.68, and the major product from the chymotrypsin digest (IV + C) had an R_F of 0.57–0.66.

Results

Recombination of Apo-b_{562} with Protoheme, Mesoheme, and Mesoheme Sulfuric Anhydride. Titration of apo-b_{562} with protoheme and mesoheme gave equivalence points which agreed within 2%. Apo-b_{562} also has a high affinity for both the mono- and bissulfuric anhydride derivatives of mesoheme, judging from the lack of curvature in the spectrophotometric titration curves obtained in preliminary experiments. However, only the monoanhydride derivative was used for reaction with apoprotein for reasons which will be discussed later. Representative titration curves for recombination of apo-b_{562} with mesoheme and mesoheme monosulfuric anhydride are shown in Figure 1. The titration curves are nearly linear, which indicates that apo-b_{562} has a high affinity for both. The blank titration with the anhydride shows a pronounced positive deviation from Beer's law. Some changes of the spectrum of the anhydride solution with time were noted, but these points indicate the initial absorbance of the solution.

[1] PTCK = tosyl-L-phenylalanylchloromethane.

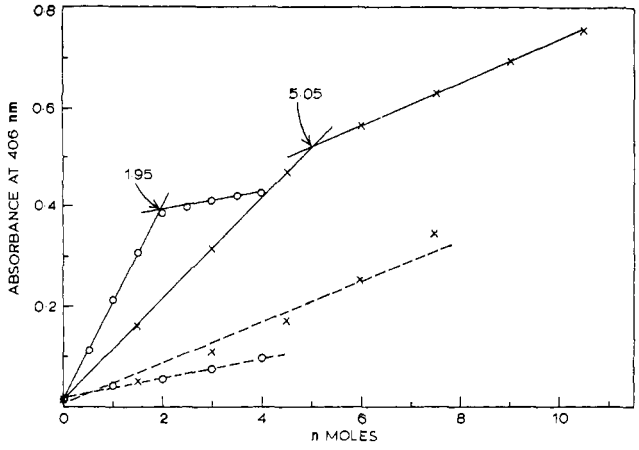

FIGURE 1: Titration of apocytochrome b_{562} with mesoheme and mesoheme monosulfuric anhydride. The mesoheme titrations are designated with circles and the titrations with anhydride are designated with crosses. The blank titrations are shown as dashed lines.

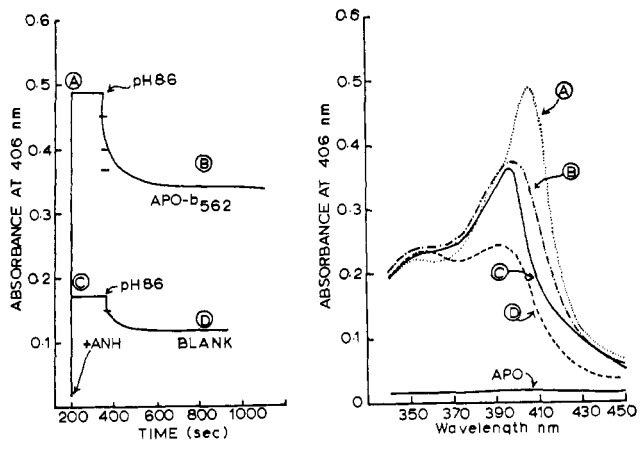

FIGURE 2: Spectral changes during reaction of mesoheme monosulfuric anhydride with apo-b_{562}. The time course of the reaction is shown on the left for apo-b_{562} (upper curve) and for the blank (lower curve). The Soret spectra recorded at the times indicated by A, B, C, and D are shown on the right.

The large difference between the equivalence points for mesoheme and monoanhydride indicates that some heme component in the anhydride sample (about 62%) is incapable of recombination with apo-b_{562}. However, the excess anhydride presents no problem if nonspecific reaction outside the heme binding site can be prevented during the reaction with apoprotein.

Spectral Changes During Reaction of Mesoheme Monoanhydride with Apo-b_{562}. The initial Soret spectrum of apo-b_{562} in the presence of 1 equiv of mesoheme monoanhydride at pH 7 is shown as curve A in Figure 2-2. The maximum is at the same position as that of mesoheme-b_{562} (406 nm), which indicates that the anhydride is initially located in the normal heme binding site. The anhydride alone gives a distinctly different spectrum described by curve C under the same conditions. When the pH is raised to 8.6, there is a rapid decrease of the absorbancy at 406 nm which is due to dilution and to a time-independent decrease of the extinction coefficient which is also observed for normal mesoheme-b_{562} at this pH. These contributions to the decrease of the absorbancy are indicated by the first and second marks on the upper curve in Figure 2-1. After the initial sharp decrease of the absorbancy at pH 8.6, there is a further slow decrease which levels off after about 10 min. The corresponding changes for the anhydride alone are shown as the lower curve in this diagram. The absorbancy of the anhydride solution also decreases slowly after the initial dilution change which is indicated by the mark. However, in this case, the magnitude of the slow decrease is only about half as great as that observed with the anhydride plus apo-b_{562}. The magnitude of the absorbancy change of the blank is indicated by the lowest mark on the upper curve. The slow change in the blank sample probably reflects both reaction with the Tris buffer and a change of the state of aggregation. A blank experiment omitting apoprotein was run under the conditions used for reaction of the anhydride with apo-b_{562}. The reaction mixture was concentrated and the heme residue was desalted on P-2 Bio-Gel. Thin-layer chromatography of the desalted heme fraction confirmed the presence of heme derivatives resulting from the reaction of anhydride with the Tris buffer.

The slow decrease of the absorbancy in Figure 2-1 is interpreted to reflect reaction of the anhydride with an amine group in the apoprotein to form an amide linkage. The rate of this "slow" decrease varied with pH. At pH 8, absorbancy changes were recorded at times up to 1 hr, whereas at pH 10, no change could be observed after the mixing time (15 sec). The final spectrum of the product at pH 8.6 has a Soret maximum at 388 nm as shown by curve B. Normal mesoheme-b_{562} has a Soret maximum at 403 nm at pH 8.6 (not shown), whereas the anhydride alone has a final maximum at 383 nm under these conditions (curve D). When the anhydride-b_{562} solution is returned to pH 7 after reaction at pH 8.6, the Soret maximum is at 402 nm instead of the initial 406 nm.

Acid–Acetone Treatment of Anhydride-b_{562}. The unreacted mesoheme was removed from the anhydride-b_{562} preparations by one of the two acid–acetone treatments mentioned in the Experimental Section. In cases where mild method I was used, the amount of unmodified apoprotein could be determined by titration with mesoheme. Generally, the sum of anhydride-b_{562} plus unreacted apo-b_{562} titratable with mesoheme was about 90% of the amount of apoprotein used for reaction with the anhydride. The fact that native apo-b_{562} was recovered in these preparations implies that the anhydride-b_{562} retains its structural integrity after the reaction treatment. The spectrum of one preparation of anhydride-b_{562} after acid–acetone treatment by method I is shown in Figure 3.

Trypsin Digestion of Anhydride-b_{562}. Before proteolytic digestion of the anhydride-b_{562} preparation, the unreacted heme was removed and the protein was denatured by the 0.12 M HCl–acetone treatment (method II) at room temperature. Trypsin treated with PTCK was used in order to prevent chymotryptic cleavages. Doubling the amount of trypsin and the time of digestion gave no significant change of the heme peptide composition, judging from electrophoresis and thin-layer chromatography results. Some of the earlier preparations of anhydride-b_{562} were made with the crude mesoheme anhydride mixture which contained predominantly the bisanhydride derivative. After digestion of these preparations with trypsin, the major heme peptide product remained

TABLE I: Summary of Purification and Yields of Anhydride-b_{562} Heme Peptides.

Sample		mμmoles	Percentage of Previous Step	Cumulative Yield (%)
Cytochrome b_{562}		883		100
Apo-b_{562}		830	94	94
Anhydride-b_{562}		832	100	94
pH 3.7 electrophoresis		612	74	69
	I	97	16	11.0
	II	156	25	17.7
pH 6.4 electrophoresis	III	36	6	4.1
	IV	121	20	13.7
	Sum	410	67	46.5
Thin-layer chromatography	I-1	17.7	4.3	2.0
	I-2	37.0	9.0	4.2
	II	111.2	27.2	12.6
	III-1	5.6	1.4	0.6
	III-2	13.6	3.3	1.5
	IV	68.0	16.6	7.7
	Sum	253.1	61.8	28.6

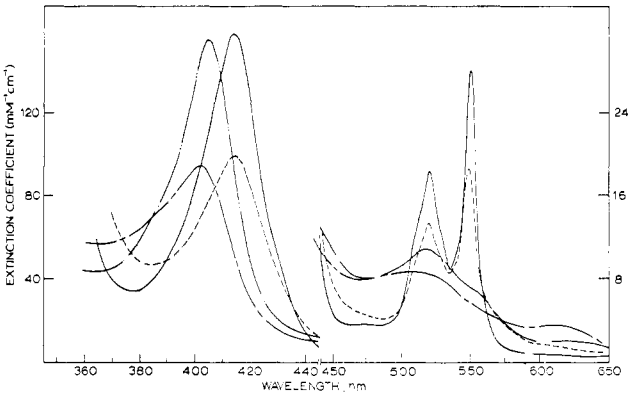

FIGURE 3: Spectral comparison of mesoheme b_{562} and anhydride-b_{562}. Concentrations were 5.0×10^{-6} and 2.5×10^{-5} M in 0.1 M phosphate buffer (pH 7.0) in the Soret and α–β band regions, respectively. (··———··) Mesoheme-b_{562} oxidized, (———) reduced, (-———-) anhydride-b_{562} oxidized, and (- - - - -) reduced.

at the origin in all of the thin-layer chromatography and electrophoresis solvents tried. Attempts to elute these bands from the paper or silicic acid with various solvents were generally unsuccessful, but the very slight amounts which could be eluted gave amino acid compositions which suggested that the heme peptides were very large and possibly were undigested anhydride-b_{562}. This chromatographic behavior might be expected for a denatured protein, so these results were interpreted to reflect a strong stabilization of the bisanhydride-b_{562} structure due to covalent attachment of the heme at two positions in the protein. This apparently makes trypsin hydrolysis difficult or impossible.

Purification of the Tryptic Heme Peptides. The methods used for purification of the tryptic heme peptides were essentially the same as the methods used for purification of the peptic heme peptides of myoglobin (Warme and Hager, 1970c). A summary of the yields for the various purification steps is given in Table I. The neutral and basic nonheme peptides had high mobilities during the electrophoresis at pH 3.7, whereas all of the heme peptides remained in a 1-in. band at the origin. The electrophoresis at pH 6.4 separated four heme peptide bands. On the anodic side of the origin there was a minor diffuse band located 0.6–2.1 in. from the origin (I) and a major band 0.1–0.6 in. from the origin (II). A minor heme band remained at the origin (III) and a second major band was located 0.3–1.3 in. on the cathodic side of the origin (IV). Several very minor heme components which had high mobilities toward the anode were also noticed, but because of their low yields, these by-products were not analyzed. Thin-layer chromatography in butanol–pyridine–acetic acid–water (15:10:3:12, v/v) was used as the final step of the purification for each of the major fractions. The peptides were spotted (10 nmoles/cm) on Eastman Chromagram sheets and developed by ascending chromatography for a distance of 14.3 cm. Fraction I gave bands at R_F 0.61–0.66 (I-1) and R_F 0.68–0.83 (I-2). There was also a trace of color at R_F 0.84–0.88 which is probably mesoheme. Fraction II gave a single band at R_F 0.55–0.62 (II). Fraction III gave two bands; one with R_F 0.51–0.58 (III-1) and another diffuse band with R_F 0.64–0.78 (III-2). Fraction IV gave a single broad band with R_F 0.65–0.77. The eluates from these factions were evaporated and aliquots were hydrolyzed with 6 N HCl as described previously (Warme and Hager, 1970c).

Identification of the Tryptic Heme Peptides from Their Amino Acid Compositions. The complete amino acid sequence of cytochrome b_{562} and the locations of the heme peptide fractions discussed below are shown in Figure 4. Some of the amino acid compositions in Table II indicate substantial levels of impurities, but the major products can be identified with certainty. Serine, glycine, and alanine contamination was especially troublesome in many cases, as was noted earlier for the peptic heme peptides of anhydride–myoglobin (Warme and Hager, 1970c). The extent of such contamination varied in several experiments, which indicates that some external source of contamination is responsible. The composition of heme peptide I-1 compares most favorably to the very long chain of residues 55–80. The proline, serine, and histidine values strongly suggest this identification. This sequence contains only two lysines; one at position 62 and the other at the carboxyl-terminal end, which must be free to account for the tryptic cleavage. Thus, the lysine at position 62 is the site of covalent attachment of the anhydride in this heme peptide. The fact that trypsin does not cleave this peptide at arginine-65 suggests that this cleavage is sterically hindered by the presence of the bulky heme group on lysine-62. However, the low yield of this heme peptide (7%), indicates that this is a comparatively minor site of reaction.

The compositions of fractions I-2 and III-2 indicate substantial contamination and no correlations with the sequence

TABLE II: Amino Acid Compositions of Anhydride-b_{562} Heme Peptides.

	Residues/Heme[a]													
Experiment:	1	b_{562}	1	1	b_{562}	1	1	1	b_{562} 102–	2	2	b_{562} 106–	2	b_{562}
Fraction:	I-1	55–80	I-2	II	46–54	III-1	III-2	IV	110	IV	IV + C	109	II + P	46–50
Lysine	2.26	2	1.15	1.96	2	1.43	0.58	1.47	2	1.03	0.93	1	0.86	1
Histidine	0.82	1	++	+		0.44	0.32	0.93	1	1.00	0.85	1		
Arginine	0.54	1	++	+			++	0.82	1	0.99	+			
Aspartic acid	5.9	5	1.69	1.03	1	0.99	0.61	0.46	1	0.88	++		+	
Threonine	0.39		0.41	1.00	1	0.52	++	+		+	+		0.77	1
Serine	1.94	2	0.46	0.26		0.31	0.30	0.26		+	+		+	
Glutamic acid	2.56	2	1.26	1.39	1	0.87	0.58	0.71	1	1.16	0.96	1	+	
Proline	1.42	2	0.64	1.81	2	1.37	++						1.69	2
Glycine	2.76	2	0.59	0.28		0.51	0.31	0.48		++	+		+	
Alanine	1.76	1	1.20	1.18	1	1.13	0.59	0.53	1	1.07	0.31		0.63	1
Valine	0.91	1	0.24											
Methionine	+	1												
Isoleucine	1.62	2	0.40	+			0.81	+			+			
Leucine	0.02	2	0.81	0.91	1	0.89	++	0.27			+		+	
Tyrosine	++		++					0.64	2	1.54	0.56	1		
Phenylalanine	1.35	2	0.36				0.30							
Yield, %	7.0		14.5	44.0		2.3	5.3	26.9						

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

of b_{562} could be made for these fractions. No attempt has been made to further purify these fractions, but their compositions suggest a heterogeneous mixture of small heme peptides which may result from nonspecific reaction with many of the 16 lysines in b_{562}. The total yield of these fractions was about 20% of the purified heme peptides.

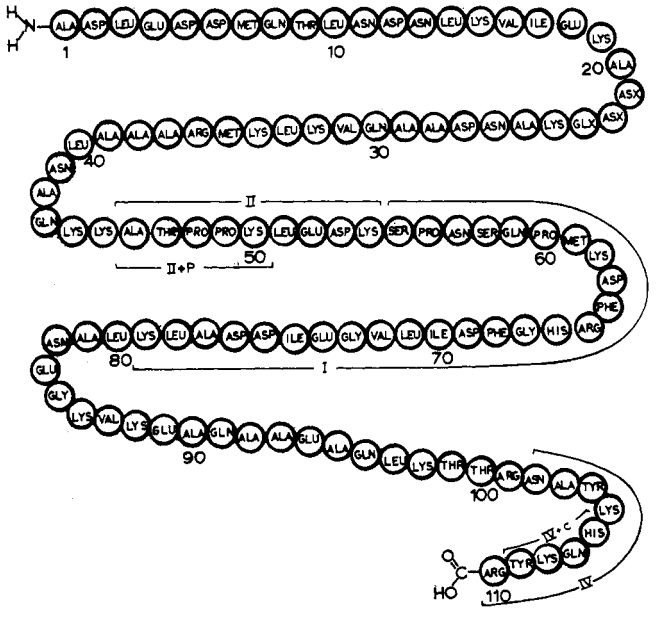

FIGURE 4: Amino acid sequence of cytochrome b_{562} showing the location of the heme peptides.

The composition of heme peptide fraction II can be unequivocally correlated with residues 46–54 in b_{562}. This sequence contains only two lysines; one at position 50 and the other at the carboxyl-terminal end, which is required for the tryptic cleavage. Thus, the lysine at position 50 is the site of heme attachment in this peptide. Long-term digestion with about 50% (w/w) pronase cleaved only the amino acids 51–54 to give heme peptide II + P (Table II). The remarkable resistance to digestion of the remainder of the sequence suggests steric hindrance by the bulky heme group. This experiment confirms the identity of heme peptide II. Although heme peptide III-1 is quite impure, its composition indicates that the major component is the same as heme peptide II. Thus, the total yield of heme peptides encompassing residues 46–54 is 46%, indicating that this is the major site of reaction of the anhydride with b_{562}.

A second major site of reaction is at lysine-108 (27%). The composition of heme peptide IV bears a resemblance to the composition of residues 102–110 in b_{562}. There are only two histidines and three arginines in b_{562}, so the presence of one residue of each in this heme peptide confirms this identification. Although a number of discrepancies will be interpreted below, the composition of the corresponding heme peptide from expt 2 (Table II) provides greater confidence in these interpretations. The low values of lysine, aspartic acid, alanine and tyrosine for the product of expt 1 can be explained by partial cleavage at lysine-105 (about 50%). This probably also accounts for the broadness of this heme peptide spot on the thin-layer plate. The extent of this cleavage varied in several

experiments, giving somewhat variable values for these amino acids. The low tyrosine values in both experiments reflect partial destruction during the acid hydrolysis, which might well be catalyzed by the presence of heme. As a further means of confirmation that lysine-108 is the site of heme attachment, a sample of heme peptide IV was digested with chymotrypsin, giving the results listed under IV + C in Table II. The composition of the product indicates that cleavage has occurred at tyrosines-104 and -109 as expected. However, the compositions of both the original heme peptide and the product of chymotryptic cleavage contain only a single lysine. The reason for this discrepancy from the predicted composition is not clear at this time. However, cleavage at both tyrosines rules out the possibility that the heme is attached by ester linkage with tyrosine.

Discussion and Conclusions

The spectrum of mesoheme-b_{562} in Figure 3 is very similar to the spectrum of normal protoheme-b_{562} (Itagaki and Hager, 1966), although the maxima are generally shifted about 10–12 nm to lower wavelengths, and the extinction coefficients are somewhat lower in most cases. A notable exception is that the extinction coefficient of the oxidized Soret band of mesoheme-b_{562} is 149, which is almost the same as that of the reduced Soret band (152) and is much larger than the oxidized Soret band extinction coefficient of protoheme-b_{562} (117). The initial Soret band of mesoheme anhydride bound to apo-b_{562} is at 406 nm before reaction at high pH. Since the mesoheme-b_{562} peak is at the same position, it is reasonable to conclude that mesoheme monoanhydride is bound by the apoprotein in the normal binding site. After reaction at pH 8.6, however, the spectrum of anhydride-b_{562} after removing the excess noncovalently bound heme is quite different from the spectrum of mesoheme-b_{562}, as shown in Figure 3. The extinction coefficients are generally lower and the spectrum indicates a mixture of high- and low-spin forms. Between pH 5 and 9, the spectrum of anhydride-b_{562} undergoes a reversible shift of the Soret maximum from 403 to 396 nm ($pK_a \sim 7.5$). The direction of the spectral shift indicates conversion from a low-spin heme complex at low pH into a high-spin complex at high pH. The spectrum of anhydride-b_{562} in 0.1 M imidazole chloride buffers gave a Soret maximum at 406 nm throughout this pH range. These observations suggest that at least some of the anhydride reacts in a way which distorts the native structure of the protein. This does not necessarily imply reaction outside the heme crevice. The difference between the angle of the amide linkage formed during the reaction and the angle for electrostatic interaction with the same amino group could be enough to cause distortion of the molecule.

The best explanation which can be offered at this time for the failure of 62% of the mesoheme anhydride molecules to recombine with apo-b_{562} is that they may be trapped in a stable aggregated form which breaks down very slowly in aqueous solution. Pyridine hemochrome analyses and thin-layer chromatography on base-hydrolyzed samples of mesoheme monoanhydride, as well as reaction of the anhydride with histidine failed to reveal any impurities of the magnitude which is required to explain this anomaly. The hydrophilic nature of the sulfuric anhydride group and the hydrophobic nature of the heme group should favor micelle formation. The positive deviation from Beer's law and the time dependence of the absorbance observed during the blank titration with the monoanhydride probably reflect a change of the state of aggregation to a smaller aggregate after dilution into the buffer. Furthermore, in experiments where excess apo-b_{562} was mixed with mesoheme monoanhydride in phosphate buffer (pH 7.0) significant increases in the absorbance were observed over a period of time greater than 1 hr. However, long-term observations of this kind are complicated by the slow hydrolysis of the anhydride in aqueous solutions which makes resolution of these processes difficult. As pointed out earlier, the excess anhydride in the reaction mixture is not a problem if precautions are taken to prevent nonspecific side reactions with external amino groups on the protein. The reaction conditions were designed to minimize such side reactions. Reaction of apo-b_{562} with mesoheme monoanhydride in dilute solution in the presence of Tris (free base) favors preferential reaction of the excess anhydride with the primary amine group of Tris. In expt 1 (see Table I), the mesoheme monoanhydride preparation was contaminated with 25% mesoheme, as shown by the histidine reaction (Warme and Hager, 1970a) test. Thus, the high yield of anhydride-b_{562} in this experiment indicates that either the apoprotein has a greater affinity for the monoanhydride than for mesoheme or that under the reaction conditions, some of the initially unbound anhydride displaces mesoheme from the heme binding site and then reacts to form a covalent bond.

A comparison of the amino acid sequence of cytochrome b_{562} with the sequence of sperm whale myoglobin determined by Edmundson (1965) reveals some homology which suggests that the structures of these proteins are similar (Figure 5). Substitutions were considered to be "conservative" if a nonpolar amino acid did not pair with a polar amino acid. Glycine, alanine, and proline were treated as being "conservative" substitutions in all cases. The wide evolutionary divergence of these proteins is reflected by the necessity of introducing many deletions in order to align the sequences as shown. However, deletions were introduced only when an increase in the number of identities would result.

In the alignment shown, the distal heme-linked histidine in myoglobin (residue 64) aligns with histidine residue 66 in b_{562}. The low-spin spectrum of b_{562} indicates that the heme has two nitrogenous-base ligands, in contrast to myoglobin. In view of the strong sequence homology in this region, it would seem that the most likely candidate for the ligand to the sixth coordination position of cytochrome b_{562} is histidine-66. The proximal histidine ligand of myoglobin is located at residue 93, whereas the only remaining histidine in b_{562} is in position 106. When these histidines are aligned, very little homology can be seen in the neighboring sequences. The homology is stronger when histidine-97 in myoglobin is aligned with histidine 106 of b_{562}. The X-ray diffraction structure of myoglobin (Kendrew, 1961) shows that only a slight displacement of the F helix would allow coordination of histidine-97 to the heme iron. A certain degree of homology between myoglobin and another *b*-type cytochrome has also been reported by Ozols and Strittmatter (1967).

The two major sites of reaction between mesoheme monoanhydride and apo-b_{562} are lysine residues 50 and 108. In view of the sequence homology between myoglobin and cytochrome b_{562}, it is of interest to discuss where these lysines might be located in the tertiary structure of b_{562} by reference to the tertiary structure of myoglobin. In Figure 5, lysine-50

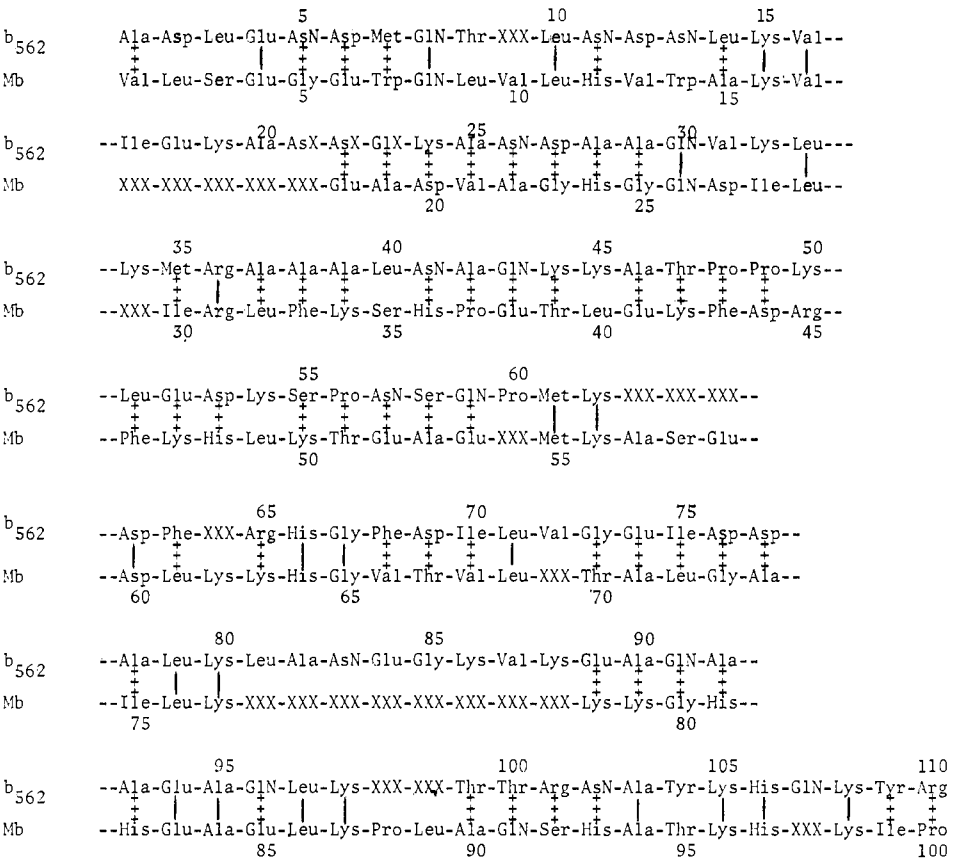

FIGURE 5: Alignment of the amino acid sequences of cytochrome b_{562} and myoglobin. Identities are connected by solid lines, "conservative" substitutions are connected by (broken) lines, and deletions are indicated by X's.

of b_{562} aligns with arginine-45 of myoglobin. In the X-ray diffraction structure of myoglobin (Kendrew, 1961), arginine-45 interacts electrostatically with one of the propionic acid side chains of the heme. The lysine at the corresponding position in horse myoglobin reacts with mesoheme monoanhydride as was shown by Warm and Hager (1970c), and it is noteworthy that lysine-50 is by far the major site of reaction in b_{562}. Since lysine-50 is preceded by two prolines, it is certain that this region of b_{562} is nonhelical, as it is known to be in myglobin. The other major site of reaction, lysine-108 in b_{562}, is so near the presumed histidine ligand that there is good reason to believe that it should be in a favorable position for reaction with the sulfuric anhydride. In fact, the proximity of these residues might very well result in distortion of the normal ligand structure when lysine-108 reacts with the anhydride, thus giving rise to the anomalous spectrum of anhydride-b_{562}. Even the minor site of reaction at lysine-62 can be rationalized in terms of the structure of myoglobin. Judging from the presence of two prolines and two serines among the seven residues preceding lysine-62, it is likely that this region is nonhelical in b_{562}. However, the corresponding lysine-56 of myoglobin is in the short D helix. The increased latitude of lysine-62 resulting from its nonhelicity might allow it to interact with one of the propionic acid side chains of the heme. In conclusion, it may be said that the sites of reaction of mesoheme anhydride with b_{562} are consistent with the hypothesis that the tertiary structure of cytochrome b_{562} is similar to that of myoglobin. However, a cautionary note should be added here. The evolutionary divergence of the amino acid sequences of these proteins has probably been accompanied by evolutionary divergence of their structures. Thus, while their gross structures may be similar, there are likely to be many differences in detail.

Since mesoheme monoanhydride reacts at two sites in cytochrome b_{562}, it is likely that both of the reactive lysines are normally involved in electrostatic interactions with the propionic acid side chains of the heme. In this connection, it is worth noting that there are two isomers of the monoanhydride, so presumably each isomer reacts specifically at one of the two different sites in b_{562}. O'Hagan (1960) showed that apohemoglobin would recombine with aethiohemin III, protohemin IX dimethyl ester, coprohemin III tetramethyl ester, and coprohemin III to form derivatives capable of reversible oxygenation. O'Hagan and George (1960) performed similar experiments on myoglobin. The conclusion from these studies was that the propionic acid side chains are not essential to the activity of these heme proteins. However, horseradish peroxidase (Maehly, 1961) and the *b*-type cytochrome from Mung bean (Schichi *et al.*, 1963) have been shown to require free propionic acid side chains in order to recombine with heme. Thus, it seems likely that other heme proteins will be found in which interaction of lysine groups from the protein with the propionic acid side chains of the heme plays a role in the binding of the heme. In these cases, reaction of mesoheme sulfuric anhydride is very likely to occur.

Judging from the fact that both apomyoglobin and apo-b_{562} have a high affinity for mesoheme monosulfuric anhydride

and both react quite specifically with the anhydride, it is likely that the techniques described here can be extended to a wide variety of heme proteins from which stable apoproteins can be prepared. The reaction conditions and purification techniques which have been described are probably generally applicable with slight modifications. In favorable cases, one or both of the ligands to the heme may be on the same chain as the lysine that reacts with the anhydride. By using various hydrolytic methods, both enzymatic and nonenzymatic, it should be possible to make a series of heme peptides of various sizes and thereby use the heme as a handle for determining the structure of the protein in the region of the heme. In cases where the heme protein has enzymatic activity, it may even be possible to isolate fragments having partial activity, and, by examination of the smallest fragments which have measurable activities, it may be possible to asses the minimal structural requirements for activity. The mesoheme anhydride method should be particularly valuable for application to very high molecular weight heme proteins, where the location of the heme in the protein structure is most obscure. The microscale purification techniques described here make this labeling method feasible on very small amounts of heme proteins which are difficult to isolate. Complete analyses were made on 1.2 μmoles of myoglobin and 0.9 μmole of b_{562}, although several trials were required in order to perfect the technique. Finally, this method may facilitate surveys of species variations of the protein structure around the heme in various heme proteins.

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