Properties of Protoporphyrin-Apomyoglobin Complexes and Related Compounds*

(Received for publication, May 3, 1967)

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SUMMARY

The conformation of the 1:1 complex of protoporphyrin IX and apomyoglobin was studied by circular dichroism, potentiometric titration, and reaction with bromoacetate. Circular dichroism studies indicate that protoporphyrin and heme similarly affect the α-helix content of apomyoglobin. The apparent number of normal imidazoles in the protoporphyrin-apomyoglobin complex, as judged by reactivity to H⁺ ion and bromoacetate, is very similar to that of myoglobin and different from that in globin. These data suggest that the conformational difference between myoglobin and apomyoglobin is determined mainly by the interaction of globin with the porphyrin and not with the heme iron. The lesser stability to acid of porphyrin complexes than of myoglobin is tentatively explained by the absence of the iron-imidazole bond in the porphyrin complexes.

Potentiometric titration of the 1:1 complex of globin with 1-anilino-8-naphthalenesulfonate indicates that this complex has the same number of normal imidazoles as does globin. These studies and those summarized above support a direct relationship between the α-helix content of myoglobin derivatives as determined by optical activity and the number of normally titratable imidazole side chains.

The photosensitivity of porphyrin-apomyoglobin complexes was shown to be due to porphyrin-sensitized photooxidation of globin. Manometric studies and amino acid analysis indicated that it is the initial oxidation of 1 or 2 histidine residues, apparently at the binding site, which leads to impairment of porphyrin binding.

Conformational differences between sperm whale metmyoglobin and apomyoglobin are manifest in differences between the two proteins in their reactivities toward H⁺ ion and bromoacetate (1) and in their α-helix content as determined by optical rotation and circular dichroism (2). We have been interested in the extent to which these conformational differences are attributable to the porphyrin alone. In a preliminary communication it was reported that the titrimetric behavior of the 1:1 complex, Pr IX-globin, of protoporphyrin IX and apomyoglobin suggests that this complex is similar in conformation to metmyoglobin (3). A marked light sensitivity of the complex was also demonstrated, but the photosensitization was not probed.

The present paper deals more generally with the optical properties and reactivity of porphyrin-apomyoglobin complexes, particularly insofar as they pertain to the effect of bound porphyrin upon apomyoglobin conformation. In addition, we have analyzed the titration of porphyrin-globin complexes in greater detail and have compared their titrimetric behavior with that of the 1:1 complex of apomyoglobin and 1-anilino-8-naphthalenesulfonate. This is of interest since ANS has been shown to compete with heme for apomyoglobin (4) and might be anticipated to have an effect similar to heme or protoporphyrin upon apomyoglobin conformation. Aspects of the photosensitization of apomyoglobin by porphyrins have also been clarified.

EXPERIMENTAL PROCEDURE

Reagents—Sperm whale metmyoglobin was obtained as a gift from Professor F. R. N. Gurd and was also purchased from Mann Research Laboratories and Pierce Chemical Company. Protoporphyrin dimethyl ester was obtained from Mann Research Laboratories and Koch-Light Chemical Company. Purity of the ester preparations was tested spectroscopically and by melting point; when necessary, further purification was achieved by chromatography on CaCO₃ followed by recrystallization (5). Free protoporphyrin was prepared from the ester by hydrolysis in 25% HCl (6), followed by recrystallization from pyridine-petroleum ether mixtures. The final product was 95 to 99% pure as judged from its Soret extinction in 25% HCl and its absorption spectrum in 0.1 N KOH. Hematopor-

* Supported by Grant HE-02739 from the National Institutes of Health and by summer stipends to R. K. from funds made available to Cornell University Medical College by United States Public Health Service General Research Grants 1-SO1-PR-05866-01 and FR-05896-04-5.

1 The chemical abbreviations used are: Pr IX-globin, the 1:1 complex of globin and protoporphyrin IX; globin, apomyoglobin; ANS, 1-anilino-8-naphthalenesulfonate; metMb, sperm whale metmyoglobin; Pr IX, protoporphyrin IX; ANS-globin, the 1:1 complex of ANS and globin.
Phyryn was obtained from Mann and was used without further purification. Hemin was obtained from British Drug Houses and from Koch-Light and was also used without further purification. The Mg$^{2+}$ salt of 1-anilino-8-naphthalensulfonate was a gift from Dr. Lubert Stryer. All other chemicals were reagent grade. Water was either glass-distilled or resin-de-ionized.

General Procedures—Apomyoglobin was prepared from metmyoglobin as previously described (1). Porphyrin-apomyoglobin complexes were prepared by addition of porphyrin in 0.1 N KOH to apomyoglobin at pH 9. Solutions were generally allowed to stand in the dark at least 1 hour before use to ensure completion of the reaction. All studies of porphyrin-globin complexes were performed in darkness.

Spectra were recorded on a Perkin-Elmer model 350 recording spectrophotometer at room temperature. Heme- and porphyrin-globin recombinations were followed on a Beckman DU spectrophotometer thermostated at 25°C. Circular dichroism studies were performed with a Jasco recording dicograph under the direction of Professor S. Beychok in the Department of Biochemistry, Columbia University College of Physicians and Surgeons. H$^+$ ion titration studies were carried out as previously described (7).

Carboxymethylation—Carboxymethylation of Pr IX-globin was performed as previously described for metMb (8), but care was taken to exclude light from the reaction mixture. After carboxymethylation was complete, excess bromoaceticate was removed by exhaustive dialysis in the dark. The resultant globin was then lyophilized and amino acid analyses were performed at Indiana University under the direction of Professor F. R. N. Gurd.

Amino Acid Analyses—In preliminary studies, the effect of extensive photooxidation by Pr IX upon apomyoglobin composition was studied by selective analysis for specific amino acids. Histidine and tyrosine were determined simultaneously by the Pauly reaction (9), and tryptophan was estimated by the Hopkins-Cole reaction (10). Estimates of tyrosine alone were made from the relative magnitudes of the ultraviolet difference spectrum between pH 7 and 12 in control and photooxidized globin. In globin, this difference spectrum results only from tyrosine (1). It was found to be unchanged by photooxidation in all studies reported here.

Amino acid analyses of globin which had been photooxidized to a limited O$_2$ consumption (see below) were performed on a Phoenix amino acid analyzer under the direction of Dr. George Frimpter of the Department of Medicine, Cornell University Medical College.

Photooxidation—Protoporphyrin-sensitized O$_2$ uptake by globin was measured manometrically at 30°C with an Aminco Warburg apparatus (15-station rotary type). A lamp with two 15-watt fluorescent tubes served as the light source. The lamp was positioned above the water bath so as to achieve an optimum reflection of light from a semicircular mirror at the bottom of the bath to the bottom of each Warburg vessel. The vessels were situated approximately 40 cm below the light source. A rapid stream of air was passed beneath the lamp to prevent warming of the manometer side arms. The vessels were shaken at 112 strokes per min at an amplitude of 4 cm.

Simultaneous studies of O$_2$ consumption and Soret absorbance were conducted as follows. In a flask shielded from the light, a stock solution was prepared consisting of globin and Pr IX in borate buffer, pH 9.1, ionic strength 0.16. Final concentrations of globin were approximately 3 x 10$^{-4}$ M. With the room minimally illuminated, each of four Warburg vessels was charged with 3.0 ml of the stock solution; 0.2 ml of 20% KOH was added to the center wells. The four manometers were positioned adjacent to one another on the water bath and between two thermobarometers. At zero time, the lamp was turned on and O$_2$ consumption as a function of time was followed in two of the sample vessels. Periodically the other two vessels were removed from the water bath; an aliquot was quickly withdrawn and the vessels were replaced. The aliquot was diluted with borate buffer, and the absorbance at 407 nm determined. The O$_2$ uptake of the latter two vessels was projected from that observed in the first two manometers. At the end of each study, the Soret absorbance of samples in which O$_2$ consumption was measured directly was compared with those used to monitor the Soret absorbance during the run. The Soret absorbance value of the two sets of samples were identical, indicating that all samples had undergone equivalent light exposure.

For preparation of globin which was photooxidized to a specific O$_2$ consumption and then analyzed, the above procedure was followed, but O$_2$ consumption was followed in all four manometers and the Soret absorbance was not periodically checked. When a total of 1.1 moles of O$_2$ had been consumed per mole of total globin, the light was extinguished and Pr IX was separated from globin in the dark by methyl ethyl ketone extraction (1) and subsequent dialysis. As a control, similar samples of globin and Pr IX were prepared simultaneously but not exposed to light, and then were analyzed similarly to the light-exposed samples. The concentrations of the resultant globins were determined by the Folin reagent, and samples of the globins were used for spectrophotometric analysis. The remainder was lyophilized; aliquots of the lyophilized material were hydrolyzed with 6 N HCl at 110°C according to the method of Moore and Stein (11) and used for amino acid analysis as described above.

RESULTS AND DISCUSSION

Spectrum of Porphyrin-Apomyoglobin Complex—Spectra of porphyrin-globin complexes relate both to the nature of the porphyrin-binding site and to the state of ionization of the bound porphyrin nucleus. In Fig. 1, the spectrum of the 1:1 complex of Pr IX and apomyoglobin at pH 9 is shown and compared with that of unbound Pr IX in chloroform-pyridine. The spectrum of Pr IX-globin is essentially identical with that observed for the complex of Pr IX and apohemoglobin (12, 13) and is strikingly similar to the spectrum of Pr IX in chloroform-pyridine. This similarity between the spectra of porphyrin-globin complexes and that of the porphyrin in organic solvents was first noted by Hill and Holden in 1926 (14); the x-ray crystal structure of metMb (15) now suggests that this effect is attributable to the nonpolar residues which line the heme (and presumably the Pr IX)-binding site.

The tetrapyrrole nucleus of free Pr IX might be expected to protonate at pH values not much below neutrality (16), with a consequent marked change in spectrum (17). However, the visible spectrum of Pr IX-globin is constant between pH 11 and 5.3 at 25°C. Since the spectrum of a cationic porphyrin nucleus differs distinctly from that of the neutral porphyrin...
in organic solvents (17), these data indicate that the porphyrin species which is bound to native globin contains an uncharged tetrapyrrole nucleus at all pH values.

Pr IX-globin begins to denature at pH 5.2 at 25° at ionic strength 0.16, and this is marked by large changes in visible and ultraviolet spectra, the latter being similar to those accompanying metMb denaturation (7). That Pr IX remains bound in some manner to acid-denatured globin is evidenced by the increased solubility of Pr IX in the presence of acid-denatured globin relative to free Pr IX. The position and relative intensity of the absorption bands of acid-denatured Pr IX-globin at pH 3 are very similar to those of Pr IX in dilute KOH (2), and differ significantly from the spectra of Pr IX derivatives in which the tetrapyrrole nucleus is cationic (17). The change in spectrum accompanying acid denaturation of Pr IX-globin is therefore similar to that occurring upon transfer of Pr IX from an organic solvent (native globin) to an aqueous environment (denatured globin), with no change in protonation of the tetrapyrrole ring.

Titration data presented previously (3) indicate that two groups on the porphyrin titrate somewhere between pH 9 and 3 in Pr IX-globin. The spectral data given here indicate that these two groups are not 2 nitrogen atoms of the pyrrole nucleus and confirm the previous assumption (3) that they must be the two Pr IX propionate side chains, the protonation of which has relatively little effect upon Pr IX spectra. This is in good accord with the lowering of the isoelectric pH of globin from 8.6 to 7.7 when Pr IX is bound, indicating the addition of approximately two negatively charged side chains. It is also in accord with the x-ray structure of metmyoglobin (15), which suggests electrostatic interactions between the heme propionate side chains and positively charged side chains of the protein. Therefore, although such a conclusion may seem intuitively obvious, these data confirm that formation of the Pr IX-globin complex involves binding of a neutral tetrapyrrole nucleus together with two propionate side chains which are uncharged at pH 8, but which protonate somewhere between pH 8 and 3.

Secondary Conformation of Porphyrin-Apomyoglobin Complexes—Preliminary optical rotation studies (3) indicated that the depth of the 233 μm trough, generally ascribed to the α-helical conformation, was the same in Pr IX-globin and in metMb. These data therefore suggested that the two proteins have the same secondary conformation. In view of the lesser influence of foreign optically active bands on circular dichroism spectra than on optical rotation, we have now studied the relative effects of the addition of porphyrins and hemin on the circular dichroism of apomyoglobin. Between 250 and 220 μm, only a single negative dichroism band, with a minimum at 222 μm, is present in apomyoglobin derivatives. The depth of this negative dichroism band at 222 μm, like its derivative 233 μm optical rotation trough, is an indication of the number of peptide bonds which are in α-helical conformation. In Table I the depth of this band is compared in globin, globin plus Pr IX, and globin plus Pr IX. The molar extinction of Pr IX in KOH is concentration-dependent, presumably because of association-dissociation phenomena. It is therefore difficult to assess the significance of the difference in apparent molar extinction between Pr IX in KOH and acid-denatured Pr IX-globin.

![Fig. 1. Spectra of free Pr IX (PIX) and Pr IX-globin (PIX-globin).](image-url)

![Fig. 2. Spectra of acid-denatured Pr IX-globin (PIX-globin) and of free Pr IX (PIX) in KOH.](image-url)

### Table I

<table>
<thead>
<tr>
<th>Sample</th>
<th>θ222 (deg·mm×cm⁻¹)</th>
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<tr>
<td>Globin</td>
<td>-16,450 ± 150</td>
</tr>
<tr>
<td>Globin + 1 eq hemin</td>
<td>-18,608 ± 300</td>
</tr>
<tr>
<td>Globin + 1 eq protoporphyrin</td>
<td>-18,541 ± 400</td>
</tr>
<tr>
<td>Native metMb</td>
<td>-19,800</td>
</tr>
</tbody>
</table>

### Notes

1. The molar extinction of Pr IX in KOH is concentration-dependent, presumably because of association-dissociation phenomena. It is therefore difficult to assess the significance of the difference in apparent molar extinction between Pr IX in KOH and acid-denatured Pr IX-globin.

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It is clear that the effect of added Pr IX upon the secondary conformation of globin is indistinguishable by this criterion from the effect of hemin. However, these studies are limited by the fact that neither of the two apomyoglobin preparations used here gave identical values of \( q \) for regenerated metMb as for the parent (native) metMb from which the globin was derived. Therefore, these data support, but do not necessarily prove, the concept that the porphyrin alone, and not the heme iron, determines the final secondary conformation of metMb.

**Reaction of Pr IX-globin with Bromoacetate**—The reactivity of myoglobin derivatives with bromoacetate is an index of their conformation (1, 8). In metMb approximately 3 fewer histidines are available for reaction with bromoacetate than in globin, in good correlation with the difference in histidine availability between the two proteins as estimated from titration studies (1, 7, 8). We have now studied the reaction of Pr IX-globin with bromoacetate under conditions similar to those used for globin and metMb except that all procedures were carried out in complete darkness (see "Experimental Procedure"). Carboxymethylation resulted in only minor changes in Pr IX-globin spectra and no apparent alteration of the light sensitivity of the complex.

Amino acid analyses of carboxymethylated Pr IX-globin are shown in Table II. These data indicate that 8 or 9 histidines react with bromoacetate in Pr IX-globin; 11 reactive histidines are present in globin (1), and a maximum of 8 may react in metMb (8). Such results support the conclusion drawn from titration studies (3) that the addition of Pr IX to globin leads to the "masking" of additional histidines. The number of additional histidines masked by Pr IX must remain tentative at 2 to 3 (bromoacetate data) or 3 (titration data below). The possibility that the \( F_8 \) histidine which is masked by heme is not masked in the presence of Pr IX, despite similarity in conformation of metMb and Pr IX-globin, has already been discussed (3). In general, therefore, the bromoacetate data support the circular dichroism studies and indicate major similarities between the structures of Pr IX-globin and metMb.

**Potentiometric Titration of Porphyrin-Apomyoglobin Complexes**—Preliminary titration analysis indicated that the number of Pr IX-globin side chains titrating in the neutral pH region prior to acid denaturation was approximately 2 fewer than in globin (see Fig. 3) and 1 more than in metMb (3). These data suggested that 2 additional histidines become masked when Pr IX binds to globin, leaving a remainder of 7 titratable histidines. Similar titration data have since been obtained for the 1:1 complex of globin with hematoporphyrin, indicating that this behavior is not unique to Pr IX globin.

We have now however analyzed the titration of porphyrin-globin complexes in terms of the Linderström-Lang model (18) by an approach previously used to analyze the titration curve of globin (1). With the assumption that \( w \) and \( \log k' \) for carbonyl, \( \epsilon \)-NH, and \( \alpha \)-NH titration are essentially identical with those in metMb and globin (1, 7), the only reasonable fit of the imidazole titration data was achieved assuming that 6, not 7, imidazoles were free to titrate with normal \( pK \). This is shown in Fig. 4, where plots of \( \log [P]/(n_{i+2}/2) \) with respect to \( Z \) are shown for different assumed values of \( n_i \), the number of titratable imidazoles in the same class. The attempt to fit the titration data with the previous assumption that \( n_i = 7 \) leads to a value for \( w \) of 0.092; this value can be

- The theoretical terms used are: \( w \), the electrostatic interaction factor; \( \log k' \), the intrinsic \( \log \epsilon \) ion association constant of a class of side chains; \( \nu_{hi} \), the number of protons bound to imidazoles which titrate within a single class; \( n_i \), the total number of imidazoles which titrate within a single class; \( Z \), the net protein charge, and \( h \), the number of protons bound per mole of protein.

### Table II

**Amino acid composition of carboxymethylated Pr IX-globin**

These analyses were performed in the laboratory of Professor Frank R. N. Gurd.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Theoretical</td>
</tr>
<tr>
<td></td>
<td>residues/mole protein</td>
</tr>
<tr>
<td>Lysine</td>
<td>19</td>
</tr>
<tr>
<td>Histidine</td>
<td>12</td>
</tr>
<tr>
<td>Asparagine</td>
<td>7</td>
</tr>
<tr>
<td>Arginine</td>
<td>4</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>7</td>
</tr>
<tr>
<td>Threonine</td>
<td>5</td>
</tr>
<tr>
<td>Serine</td>
<td>6</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>10</td>
</tr>
<tr>
<td>Proline</td>
<td>4</td>
</tr>
<tr>
<td>Glycine</td>
<td>11</td>
</tr>
<tr>
<td>Alanine</td>
<td>17</td>
</tr>
<tr>
<td>Valine</td>
<td>8</td>
</tr>
<tr>
<td>Methionine</td>
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</tr>
<tr>
<td>Isoleucine</td>
<td>9</td>
</tr>
<tr>
<td>Leucine</td>
<td>18</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3</td>
</tr>
<tr>
<td>Phenyalanine</td>
<td>6</td>
</tr>
<tr>
<td>1-Carboxymethylhistidine</td>
<td>0</td>
</tr>
<tr>
<td>3-Carboxymethylhistidine</td>
<td>0</td>
</tr>
<tr>
<td>Dicarboxymethylhistidine</td>
<td>0</td>
</tr>
</tbody>
</table>

**Fig. 3.** Potentiometric titration of globin, Pr IX-globin (PIX-globin), and the 1:1 complex of globin and ANS at 25°C and ionic strength 0.16. For convenience, all curves are adjusted to \( h = 0 \) at \( pH 8.0 \), the isionic \( pH \) of globin.
shown to increase with increases in the assumed value of $n_i$. Such a value for $w$ is unreasonably high, as it can be assumed that $w$ for imidazole titration in Pr IX-globin should lie between 0.05 and 0.066 at ionic strength 0.16: the corresponding values in metMb and globin, respectively. Alternatively, the assumption that $n_i = 6$ gives the expected value of 0.004 for $w$. The derived value of log $k'$ for the imidazoles in this class in Pr IX-globin is 6.80 and may be compared with values of 6.70 and 6.76 in metMb and globin, respectively (1).

The apparent number of normally titratable imidazoles in Pr IX-globin is therefore the same as in metMb (7). However, the assumption that the same parameters as in metMb govern the titration of all side chains does not completely explain the titration of native Pr IX-globin. The plot in Fig. 4 is linear with these assumptions only until a change of $-5$, corresponding to pH 6, is attained. Spectroscopically, acid denaturation of Pr IX-globin is not manifest until pH 5.2 at ionic strength 0.16, so that nonlinearity is not obviously associated with denaturation, nor is the premature divergence from linearity corrected by assuming a larger number of "normal" imidazoles or any reasonable change in the average carboxyl $pK$. It can be explained either by assuming that some conformational changes do occur prior to spectroscopic denaturation, or that two groups which have effective $pK$ values below 4 in metMb have apparent $pK$ values close to 5.7 in Pr IX-globin, or by both assumptions. These groups might include the F$_3$ histidine (3) or the two Pr IX carboxyls, the $pK$ of which might be higher in the absence than in the presence of trivalent iron.

**Stability of Porphyrin-Globin Complexes to Acid**—The $pK$ region of acid denaturation of Pr IX-globin and hematoporphyrin-globin is shifted approximately 0.7 pH unit higher than in native or regenerated metMb (3, 7). This is evidenced in Fig. 3 by the abrupt change in titration slope of Pr IX-globin at approximately pH 5.2; a similar change in metMb titration under these conditions is not manifest until approximately pH 4.5. Since the conformations of heme-globin and porphyrin-globin complexes appear similar, the difference in their stability is most readily attributed to differences between Pr IX and heme in the enthalpy of binding to globin. The most probable source of this difference is the presence of the imidazole-iron bond in the heme complexes only. It is of interest that the difference in $pH$ stability of heme- and porphyrin-globin complexes stands in contrast to the similarity in the rate of interaction of hemes and porphyrins with globin (19). Conceivably the iron-imidazole bond is formed subsequent to the transition state during globin-heme combination.

**Potentiometric Titration of Apomyoglobin-ANS Complex**—The similarity in conformation between porphyrin- and heme-globin complexes lends weight to the thesis that the difference in conformation between globin and metMb is determined mainly by hydrophobic interactions between porphyrin and globin (2). Competition between heme and ANS upon binding to globin (4) may indicate the proximity or identity of the heme and ANS-binding sites, and this is supported by fluorescence studies of the ANS-globin complex (4). In addition, interactions between ANS and globin may be largely hydrophobic (4). The question arises whether ANS induces the same conformational change in globin as do heme and Pr IX. Stryer's data (4) indicate that binding of ANS to globin does not alter globin levorotation at 233 m$\mu$ and so presumably has no effect on $\alpha$-helix content. We have now titrated the 1:1 complex of globin and ANS in order to observe the effect of ANS on histidine reactivity. In Fig. 3, the titration curve of the 1:1 apomyoglobin-ANS complex is shown relative to that of globin and porphyrin-globin. In contrast to the effect of Pr IX on the titration curve of globin, binding of ANS leads to essentially no decrease in the number of groups titrating in the neutral $pH$ region prior to acid denaturation. Nor is the number of titratable side chains exposed upon acid denaturation increased in ANS-globin as in the porphyrin-globin complexes (3). These data indicate that ANS does not alter histidine reactivity in globin as does bound porphyrin.

The lack of change in histidine reactivity brought about by ANS parallels its lack of apparent effect on $\alpha$-helix content and may be contrasted with the effect of porphyrins and heme on both histidine reactivity and secondary conformation. These results support a direct correlation between $\alpha$-helix content and histidine reactivity in myoglobin derivatives. In addition, they caution against strict identification of the ANS-binding site with the heme- and porphyrin-binding site, since the conformations to which ANS and heme bind are different.

**Effect of Light on Porphyrin-Apomyoglobin Complexes**—Combination of porphyrins with apomyoglobin can be followed spectrophotometrically by the increase in Soret band intensity attendant to binding. If a typical Pr IX-apomoglobin combination is followed spectrophotometrically in even dim light, the complex is observed to form immediately in 1:1 ratio and then progressively dissociate with time (Fig. 5). No dissociation of the complex is observed if samples are kept in the dark. The dissociation of the complex in light is not due to the light sensitivity of Pr IX itself, since (a) Pr IX exposed to equivalent light in the absence of globin can still combine with native globin and (b) residual globin from the light-altered Pr IX-apomoglobin complex can no longer combine with heme, an observation first made by Hill and Holden in their studies of the hematoporphyrin-globin complex (14). Therefore it is the globin of the Pr IX-apomoglobin complex which is altered in the presence of light, so that its Pr IX- and heme-binding

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**Fig. 4.** Plot of pH + log $[\omega/(n_i - \omega)]$ with respect to $\omega$ for titration curves and corrected for the titration of $\epsilon$-NH$_2$, $\alpha$-NH$_2$, and carboxyl side chains assuming values of pH for these residues of 10.3, 7.8, and 4.48, respectively, and $\omega = 0.05$. 

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**Table 4**

<table>
<thead>
<tr>
<th>$n_i$</th>
<th>log $k'$</th>
<th>$\omega$</th>
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<tbody>
<tr>
<td>6</td>
<td>6.80</td>
<td>0.054</td>
</tr>
<tr>
<td>7</td>
<td>6.74</td>
<td>0.092</td>
</tr>
</tbody>
</table>

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**Graph 4**

- Figure 4 shows a plot of pH + log $[\omega/(n_i - \omega)]$ with respect to $\omega$ for titration curves and corrected for the titration of $\epsilon$-NH$_2$, $\alpha$-NH$_2$, and carboxyl side chains assuming values of pH for these residues of 10.3, 7.8, and 4.48, respectively, and $\omega = 0.05$. The graph illustrates the effect of ANS on histidine reactivity in globin as compared to the effect on porphyrin-globin complexes.
site is directly or indirectly destroyed. This is consonant with the fact that Pr IX is a known photosensitizer (20) and, indeed, we have observed that the rate of fall in Soret band intensity is first order in oxygen pressure.

The effect of light on the Pr IX-apomyoglobin combination curve with time (Fig. 5) indicates that loss of Pr IX-binding affinity occurs most readily in globin molecules to which Pr IX is specifically bound. This is shown by the fact that the rate of fall in Soret band intensity increases with increasing Pr IX to globin ratio until a Pr IX to globin ratio of 1:1 is reached, and then remains constant. Thus, the rate of fall in Soret band intensity is not proportional to the concentration of total Pr IX or free Pr IX, but is proportional to the concentration of the Pr IX-globin complex. The important role of specifically bound Pr IX in damaging the globin-binding site was also seen in studies of the effect of free Pr IX on the Soret of metmyoglobin. Here, exposure to light results in a much slower rate of fall than with Pr IX-globin. Since the conformations of metmyoglobin and Pr IX-globin appear the same, this must mean that the heme-binding site is most sensitive to Pr IX when Pr IX is directly attached to it.

It is relevant to studies below to note that, in the 1:1 Pr IX-globin complex, the fall in Soret band intensity upon exposure to light begins immediately. However, from Fig. 5 it is apparent that at Pr IX to globin ratios less than 1, there is a lag period before the fall in the Soret band begins. The duration of the lag period increases with increasing globin to Pr IX ratio. Since the lag period is accompanied by O2 consumption, it can be attributed to binding of Pr IX liberated from photooxidized globin to excess native globin.

4 E. Breslow, R. Koehler, and A. Girotti, unpublished observations.

Preliminary Identification of Photooxidized Residues—Photosensitization by Pr IX appears to occur through the following mechanism. Photoexcited Pr IX molecules transfer energy to O2, exciting O2 to a singlet state; photooxidation of susceptible organic molecules proceeds by attack of singlet O2 (21). Since the probability that a given amino acid will be oxidized increases with its proximity to the point of origin of the excited O2, the special lability of the heme-binding site can be interpreted in terms of preferential oxidation of residues immediately at the binding site when Pr IX is attacked. Free Pr IX should photosensitize more indiscriminately than bound Pr IX around the globin (or metMb) periphery, and is thus less efficient at destroying critical side chains. It therefore seemed of interest to identify those residues on apomyoglobin, the photooxidation of which critically leads to impairment of Pr IX and heme binding ability.

The extent of O2 consumption attending photooxidation is an index of the number of amino acids oxidized. In initial attempts to determine the number of residues photooxidized during loss of Pr IX binding, the rate of O2 uptake and the rate of change in Soret band intensity were followed simultaneously when a 1:1 mixture of Pr IX and globin was exposed to light. Under these conditions, 3.5 moles of O2 were consumed at the midpoint of the Soret band fall, and O2 uptake continued unabated when the fall in Soret was complete. Amino acid analyses (see “Experimental Procedure”) of a sample of Pr IX-globin in which the Soret had completed its fall showed a loss of most of the histidine residues, a possible slight loss of tryptophan, and no loss of tyrosine. These data indicated that liberation of free Pr IX from the 1:1 Pr IX-globin complex leads to indiscriminate oxidation of a number of globin side chains before the fall in Soret band is complete.

In order to minimize photooxidation of globin by unbound Pr IX, subsequent studies were done under conditions of low Pr IX to globin ratio. Under these circumstances, if Pr IX released from photooxidized globin binds to excess native globin more rapidly than it will photosensitize residues not proximal to the binding site, little nonspecific photooxidation should occur until a decrease in Soret absorbance signals that the concentration of native globin has fallen below that of Pr IX.

In Fig. 6, the course of O2 uptake and Soret absorbance change is shown for a 1:10 mixture of Pr IX and globin when exposed to light. Absorbance at the Soret remains constant until 1.2 moles of O2 per mole of total globin have been consumed, and then falls linearly. Inasmuch as the O2 consumption at the onset of the Soret band fall corresponds to the amount used to photooxidize 90% of the total globin present, these data indicate that 1.3 moles of O2 were consumed per mole of globin so photooxidized that its ability to bind Pr IX was impaired. In similar experiments in which the ratio of Pr IX to globin was 1:5, 1.6 moles of O2 were consumed for each mole of globin in which the Pr IX-binding site was damaged. Since it may be assumed that a minimum of 1 mole of O2 is consumed per mole of amino acid photooxidized (22), the observed O2 consumption suggests that impairment of Pr IX binding is accompanied by the oxidation of 1 to 2 residues. The observed number of residues photooxidized

5 An alternative mechanism of photosensitization has been postulated by Gollnick and Schenck (22). This mechanism would in this case involve oxidation by an activated porphyrin-O2 complex. However, it appears less likely than oxidation by singlet O2 (21) and does not affect the conclusions presented here.
oxidized is higher than the minimum number necessary for alteration of the binding site to the extent that noncritical residues were photooxidized. Therefore, it is possible that the photooxidation of a single residue, most likely at the binding site, leads to loss of Pr IX binding.

The nature of the first residue photooxidized was determined by amino acid analyses in separate studies. In these studies, photooxidation of globin in the presence of 0.1 eq of Pr IX was followed manometrically until an uptake of 1.1 moles of O₂ per mole of total globin was observed. The light was then extinguished, and the Pr IX was removed in the dark (see “Experimental Procedure”). Samples of the resultant photooxidized globin were taken for spectrophotometric analysis of tryptophan; the remainder was lyophilized and used for amino acid analysis. As a control, another sample of globin and Pr IX, which had never been exposed to light, was treated and analyzed identically.

Ultraviolet spectra of the photooxidized globin showed that its spectrum between 250 and 300 nm was essentially identical with that of native globin. Well, Gordon, and Buchert (23) have shown that the spectrum of tryptophan in this wave length region is almost obliterated by photooxidation. Since each globin tryptophan contributes approximately 30% of the total globin extinction in this wave length region, any photooxidation of tryptophan should be readily discernible. These data therefore indicate that tryptophan is not the first amino acid oxidized.

Apart from tryptophan, the potentially photooxidizable amino acids of globin are histidine, tyrosine, and methionine (23). Analyses for these residues, along with some nonoxidizable amino acids as internal standards, are shown in Table III. In agreement with our previous analyses of more radically photooxidized globin, the data indicate that tyrosine is unaffected by photooxidation to this stage. The only amino acid demonstrably affected by this photooxidation is histidine; the calculated loss of 1.6 histidines per globin is slightly greater than the observed O₂ consumption of 1.1 moles would suggest. Therefore, it appears that the first residue photooxidized is a histidine.

The mechanism of photosensitization by Pr IX suggests that the first residues photooxidized should lie in the vicinity of bound Pr IX. The observation here that 1 or 2 histidines are photooxidized prior to release of Pr IX from its specific binding site on globin is in accord with this inasmuch as 3 histidines, E7, F8, and FG3, are known to be immediately at the heme-binding site in crystalline metMb (15). Although F8 is the Fe³⁺ ligand in metMb, the mechanism by which oxidation of any of these imidazoles leads to loss of affinity for both heme and Pr IX is unclear. Speculation is premature without positive identification of the photooxidized histidine (or histidines) and an assessment of the homogeneity of the photooxidation product. One possibility, however, is that photooxidation is accompanied by conformational changes. Alternatively, the loss of heme and Pr IX binding may result from the nature of the photooxidation product, which might sterically impede binding of the tetrapyrole nucleus or destroy the necessary hydrophobic character of the heme and Pr IX environment.

Acknowledgments—The authors wish to express their appreciation to Miss Priscilla Anderson and Mrs. Denise Pleckaitis for their excellent technical assistance, to Professor F. R. N. Gur with gifts of myoglobin and amino acid analysis of carboxymethylated protoporphyrin-globin, to Professor Sherman Beychok of Columbia University for use of the amino acid analyzer, and to Dr. George Frimpter of the Department of Medicine for use of the amino acid analyzer.

REFERENCES

Properties of Protoporphyrin-Apomyoglobin Complexes and Related Compounds
Esther Breslow, Robert Koehler and Albert W. Girotti


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