Photooxidation of the Protoporphyrin–Apomyoglobin Complex†

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ABSTRACT: The 1:1 complex of protoporphyrin IX (a photosensitizer) and sperm-whale apomyoglobin was irradiated with visible light at pH 9.2. Oxygen consumption was followed manometrically. After the consumption of 1 mol of oxygen/mol of protein, the reaction was stopped, porphyrin extracted, and the photooxidized protein studied in various ways. Polyacrylamide gel electrophoresis disclosed that the reaction product was no less homogeneous than an unphotooxidized control. Spectrophotometric titration of the photooxidized protein with protoporphyrin revealed that the 1:1 stoichiometry is retained, although the binding is somewhat weakened. Similar results were obtained with hemin, the natural prosthetic group. Amino acid analysis of photooxidized globin indicated that no photooxidizable amino acids except histidine (1 out of 12 in the protein) were modified. Tryptic peptide maps of photooxidized globin were prepared and analyzed simultaneously with maps of native globin. The ninhydrin color intensity of one peptide spot was markedly reduced on the photooxidized globin maps. This peptide was eluted from maps of native globin, subjected to amino acid analysis, and identified as Lys^{78} through Lys^{96} in the myoglobin sequence. Our results indicate that His^{93} in this sequence is highly photoreactive by virtue of its proximity to the porphyrin ring in the protoporphyrin–apomyoglobin complex. This correlates with the fact that His^{93} is known to interact directly with heme iron in crystalline myoglobin (Kendrew, J. C. (1962), *Brookhaven Symp. Biol. 15*, 216). These results demonstrate the feasibility of using protoporphyrin as a site-specific photosensitizer for probing active sites in hemoproteins.

The interaction of protoporphyrin IX and sperm-whale apomyoglobin was first studied in detail by Breslow and Koehler (1965) and by Breslow *et al.* (1967). By physicochemical means these investigators found that proto[1] can combine stoichiometrically with globin to form a complex that is structurally very similar to the natural hemoprotein. In contrast to the hemoprotein, however, proto–globin was found to be extremely photolabile. This effect was shown to be directly attributable to the photosensitized oxidation of globin by proto. It was postulated that the active species in this reaction is singlet molecular oxygen; recent evidence has lent support to this idea (Dalton *et al.*, 1972). It was pointed out by Breslow *et al.* (1967) that photooxidation of the proto–globin complex, when carried out under appropriate conditions, might result in the preferential modification of susceptible residues within the porphyrin binding pocket. Experiments to test this possibility were described. When proto–globin was subjected to limited irradiation with visible light, only one or two histidines, and no other photooxidizable residues in the protein (tryptophan, tyrosine, or methionine) were modified before proto binding was significantly weakened. Although the sequence position of the rapidly reacting histidine(s) was not determined, the results suggested that the residue(s) should lie in close proximity to the porphyrin ring in the proto–globin complex.

In the present article a more extensive investigation of the sensitized photooxidation of proto–globin is described. The major objective of this work has been to analyze the photooxidation product and to identify the sequence positions of any unusually photolabile residues. We have been able to accomplish this and to deduce that the photooxidation of the proto–globin complex can, in fact, occur in a highly selective or site-directed manner. On the basis of these observations with myoglobin as a model system, we feel that the photochemical approach described has potential as a means of probing the heme binding environment of other hemoproteins.

Experimental Section

Materials. Sperm-whale metMb was obtained from Miles-Seravac laboratories. This material was found to contain one major and at least three minor components on polyacrylamide electrophoresis at pH 9.5, in agreement with previous reports on myoglobin microheterogeneity (Raymond, 1964). Three components were resolved on carboxymethylcellulose chromatography (Hardman *et al.*, 1966). The major component accounted for more than 90% of the material, so purification of the commercial protein was deemed unnecessary for the purposes of our work. Recrystallized hemin and protoporphyrin IX dimethyl ester were purchased from Schwarz/Mann and used without further purification. Synthetic His-Lys was supplied by Sigma Chemical Co. Tos-$PheCH_2Cl$-trypsin was obtained from Worthington Biochemicals Corp. All other chemicals were reagent grade, and all solutions were prepared with glass-redistilled water.

Globin Preparation. Globin was prepared from deionized metMb by the procedure of Breslow (1964). The hemin-extracting solvent was methyl ethyl ketone (Teale, 1959). Globin preparations usually contained less than 1 mol % of metMb, according to spectral analysis. To minimize precipitation of the apoprotein on standing, the following steps were taken: (a) methyl ethyl ketone was redistilled before use; (b) dialysis tubing (Union Carbide Corp.) was pretreated according to McPhie (1971). Stock solutions of globin (20–30 mg/ml) were stored at 4° and used for periods up to 3 weeks. Any sediment

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[1] Abbreviations used are: proto, protoporphyrin IX; metMb, sperm-whale metmyoglobin; globin, apomyoglobin; Tos-$PheCH_2Cl$, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone; globin(1-O_2), globin-(2-O_2), globin photooxidized to an uptake of 1 and 2 molar equiv of oxygen, respectively, in the presence of 1 molar equiv of proto.

that appeared during this time was removed by centrifugation, and the protein concentration redetermined (see below).

Protein Concentrations. MetMb and globin concentrations were determined spectrophotometrically using published extinction coefficients. At pH 7.0 (0.1 M phosphate buffer) a value of 153 mM^{-1} cm^{-1} at 409 nm was used for metMb (Hanania *et al.*, 1966); 15.8 mM^{-1} cm^{-1} at 280 nm was used for globin (Stryer, 1965). Protein concentrations were also determined by the Folin–Lowry method (Lowry *et al.*, 1951), and by a microbiuret reaction (Goa, 1953), using appropriate standard curves in each case.

Absorbance values were recorded at room temperature with a Gilford Model 240 spectrophotometer.

Preparation of Proto. Protoporphyrin IX dimethyl ester was purchased from Schwarz/Mann. Proto was prepared from the ester by a slight modification of the hydrolysis procedure of Grinstein (1947). All steps were carried out in minimal light. The ester, 100 mg, was dissolved with 50 ml of 25% (w/v) HCl and stirred for 5 hr at room temperature. After adjusting the solution to approximately pH 4 with 6 N NaOH, enough 1 M sodium formate buffer, pH 3.8, was added to bring the final buffer concentration to 0.2 M. Proto was extracted into peroxide-free ether and the combined extracts were washed with water and taken to dryness on a rotary evaporator. The recovered powder was stored over P_2O_5 at $-15°$.

The purity of the product was judged from its visible spectrum in 2.7 N HCl. Absorption peaks were found at 409, 557, and 601 nm, with extinction coefficients of 263, 13.5, and 5.3 mM^{-1} cm^{-1}, respectively, in agreement with published values (Falk, 1964).

Reconstitution Studies. The ability of globin to recombine with hemin or to combine with proto was assessed by spectrophotometric titration, essentially as described by Breslow (1964). Reactions with proto were conducted in the dark. Stock solutions of proto or hemin were prepared by dissolving the material in 0.1 N NaOH and quickly diluting to a final concentration of alkali between 1 and 5 mM. Fixed concentrations of globin in 0.2 M borate buffer, pH 9.2, were incubated with various concentrations of porphyrin for 1 hr at 4°. The changes in Soret absorbance were recorded as a function of the molar ratio of porphyrin to globin.

Photooxidation Procedure. Photooxidation reactions were monitored by oxygen uptake on a Warburg apparatus equipped with a water bath with an acrylic plastic bottom (Gilson Medical Electronics, Model RWBP-3). All reactions were carried out at 25° with appropriate thermobarometer corrections. A 30-W reflector spot light positioned ~14 cm below the bottom of each reaction vessel served as the light source. Variable light intensity was achieved with a Powerstat. The vessels were shaken at 125 strokes/min and an amplitude of 5 cm. Stock proto–globin (1:1) solutions in 0.2 M borate buffer, pH 9.2, were prepared in the dark. With the room lighting as dim as possible, each of five vessels was filled with 3.0 ml of stock solution (30–40 mg of protein/vessel); 0.2 ml of 20% KOH was added to each center well for CO_2 absorption. The lights were turned on to start the reaction and O_2 consumption was followed continuously until a specific level had been reached. (Light intensity was adjusted so that an uptake of 1 mol of O_2/mol of globin required approximately 15 min.) At this point the lights were extinguished and proto was separated from globin by methyl ethyl ketone extraction; the protein was dialyzed as described by Breslow (1964). Samples of the photooxidized protein were removed for spectral and electrophoretic analysis; the remainder was lyophilized and stored at $-15°$.

Polyacrylamide Electrophoresis. Analytical polyacrylamide gel electrophoresis was performed at room temperature with a Buchler apparatus according to the procedure of Davis (1964). The acrylamide monomer concentration was 7.5% (w/v). Electrophoresis was carried out at 3 mA/tube with a Tris-glycine buffer, pH 8.3. The gels were stained with Amido Black 10B.

Tryptic Hydrolysis and Peptide Mapping. To prepare tryptic peptides, solutions of globin or photooxidized globin (10 mg/ml) were heated on a steam bath for 20 min, cooled, and treated with 1.25% (w/w) Tos-PheCH_2Cl-trypsin. Hydrolyses were carried out at 38° under nitrogen, and pH was maintained at 8.5 by the periodic addition of standardized 0.1 N NaOH. After 24 hr the consumption of alkali corresponded to approximately 50% bond splitting after basic amino acids, in agreement with previous findings (Edmundson and Hirs, 1962). Reactions were then terminated by lowering the pH to 6.4 with 0.1 N HCl. A gelatinous precipitate, the so-called "core," usually formed at this stage; it was removed by centrifugation and the supernatant containing the soluble peptides was lyophilized.

Maps of the soluble peptides were prepared by a modification of the procedure of Baglioni (1961). Peptide solutions were standardized by the Folin–Lowry method (Lowry *et al.*, 1951). Samples of 1.5–3.0 mg of peptide material were applied to Whatman No. 3MM paper for separation. Descending chromatography was carried out first with the solvent system pyridine–isoamyl alcohol–water (35:35:30, v/v). High voltage electrophoresis was accomplished with a Gilson Electrophorator (Model D), using the pH 6.4 buffer system pyridine–acetic acid–water (25:1:474, v/v). Running conditions were usually 3000 V for 100 min. After drying, the maps were sprayed either with ninhydrin (0.2% in ethanol) or with the Pauly reagent (Fraenkel-Conrat and Singer, 1956) to expose histidine- and/or tyrosine-containing peptides. Peptides for amino acid analysis were detected on maps sprayed with 0.01% ninhydrin. These faintly stained spots were cut out and eluted with water. The eluates were passed through a sintered glass filter and concentrated on a rotary evaporator, and the recovered peptides were subjected to acid hydrolysis (see below).

Amino Acid Analyses. The amino acid content of globin before and after photooxidation, and of tryptic peptides of globin, was determined by the procedure of Moore and Stein (1963). All samples were hydrolyzed in 5.7 N HCl for 24 hr at 110° in sealed evacuated tubes. Hydrolysates were chromatographed on a Beckman/Spinco Model 120B analyzer.

Results

The visible spectrum of globin in the presence of 1 equiv of proto at pH 9.2 is shown in Figure 1. Before exposure of this solution to visible light, the peak positions and extinction coefficients are very close to those previously reported for the 1:1 proto–globin complex (Breslow *et al.*, 1967). The complex was irradiated and oxygen uptake was followed manometrically as described (see Experimental Section). Reactions were stopped after the consumption of 1.01 ± 0.02 and 2.02 ± 0.02 mol of oxygen/mol of globin, and samples were removed for spectral analysis. As shown in Figure 1, gradual spectral changes are observed as photooxidation takes place. Although no shift in position of the Soret band (407 nm) and four minor bands (504, 543, 570, and 623 nm) is evident after 2 mol of oxygen has been consumed, Soret absorbance has decreased 15% while the absorbance of each minor peak has

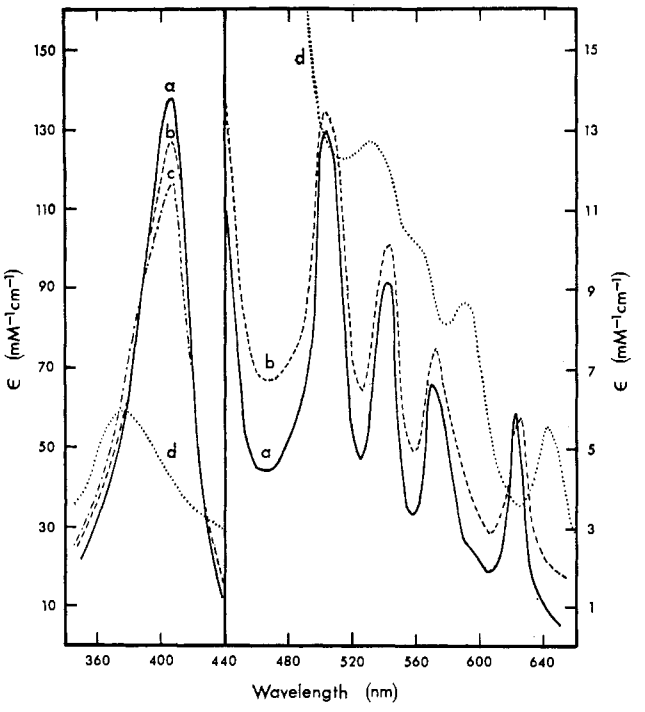

FIGURE 1: Visible spectra of (a) unphotooxidized proto–globin; (b) proto–globin photooxidized to 1-O_2; (c) proto–globin photooxidized to 2-O_2; and (d) free proto in 0.32 M sodium borate buffer, pH 9.2.

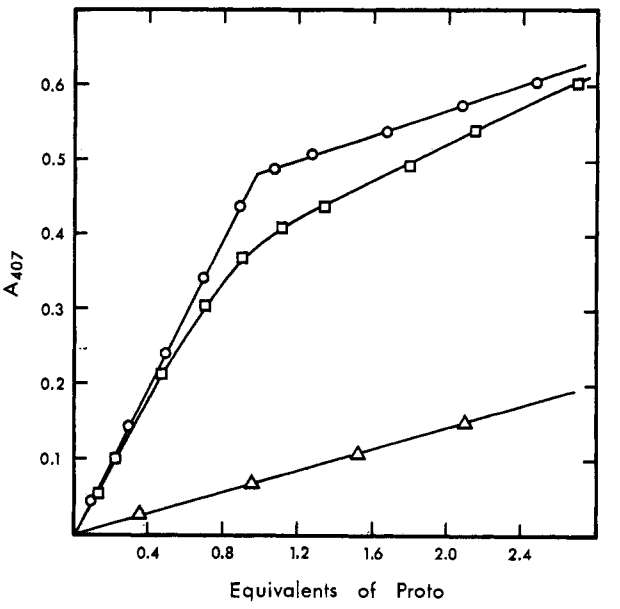

FIGURE 2: Spectrophotometric titration of native globin (○); globin photooxidized to 1-O_2 (□); and globin-free buffer (△). Protein concentrations: 3.6 μM in 0.2 M sodium borate buffer, pH 9.2.

increased slightly. These changes suggest that limited photooxidation (1–2 O_2) affects the interaction of proto and globin. Although binding of proto might be weakened, there appears to be no large scale release of the porphyrin from the protein surface, since the spectrum is far different from that of free proto. That the reaction is truly light induced is demonstrated by the fact that control solutions of proto–globin shielded from the light neither consumed oxygen nor showed time-dependent spectral changes. It is unlikely that the spectral shifts of irradiated proto–globin are attributable to self-destruction of proto. Although the spectrum of proto extracted from photooxidized proto–globin was not examined, control solutions of proto irradiated in the absence of globin consumed no oxygen and showed no spectral changes. Photooxidized globin was tested for its ability to recombine with freshly prepared proto by spectrophotometric titration at 407 nm. A typical titration curve of globin(1-O_2) is compared with that of native unphotooxidized globin in Figure 2. Native globin is protein that had no previous association with proto. It was found that protein isolated from a control 1:1 proto–globin solution incubated in the dark had titration characteristics identical with those of native globin, *i.e.*, a sharp end point at 1 equiv of proto and a corresponding extinction coefficient of ~135 mM^{-1} cm^{-1}. In the case of globin(1-O_2) the 1:1 stoichiometry appears to be maintained, but curvature in the plot suggests that proto binding is somewhat weaker after photooxidation. The dissimilarity in the control and 1-O_2 titration curves emphasizes the fact that photooxidation of proto–globin affects the protein and not the porphyrin group. Titration of globin(1-O_2) with hemin (not shown) gave essentially the same results as with proto, which indicates that the presence of iron does not stabilize the interaction of porphyrin and photooxidized globin. When globin was photooxidized to a consumption of 2 mol of oxygen and beyond, there was a progressive shift in the titration curve toward that of the globin-free blank (bottom curve, Figure 2). Eventually, therefore, proto-sensitized photooxidation of globin can completely obliterate the recombination of the protein with proto itself or with the natural prosthetic group, hemin.

The effect of limited photooxidation on the electrophoretic properties of globin was assessed by polyacrylamide gel electrophoresis. Gel patterns of native globin and globin(1-O_2) are shown in Figure 3. The band pattern of untreated globin deserves some comment. At least three major bands are observed. This is consistent with the fact that commercial sperm-whale myoglobin exhibits microheterogeneity (Raymond, 1964). The three bands collectively have a lower mobility toward the anode than the bands of metmyoglobin (not shown). This follows from the fact that the isoionic points of globin and metmyoglobin are 8.63 and 7.86, respectively (Breslow, 1964). Although multiple bands were always observed for metmyoglobin and for the globin derived from it, the actual number of bands was always lower for the apoprotein. The explanation for this is not clear, although the following possibilities exist: either precipitation of the missing material during heme removal or band overlap resulting from the apoprotein's lower mobility. As can be seen in Figure 3, the overall electrophoretic pattern of globin(1-O_2) is virtually the same as that of native globin with the exception of a small amount of band smearing. When a number of gels was examined, it was found that the photooxidized material as a whole was slightly more anionic than the nonphotooxidized. This is consistent with the charge properties observed for other photooxidized proteins, *e.g.*, pancreatic ribonuclease (Kenkare and Richards, 1966). The fact that globin(1-O_2) is effectively no more heterogeneous than native globin supports our contention that photooxidation has occurred in a highly selective manner rather than at random over the protein surface. We believe that this specificity results from the high affinity of proto for its binding "pocket" during the early stages of the photoreaction. This condition gradually changes if photooxidation is allowed to continue beyond the 1-O_2 stage. In one experiment globin(4-O_2) was prepared and examined electrophoretically. Only two faint bands in a broad

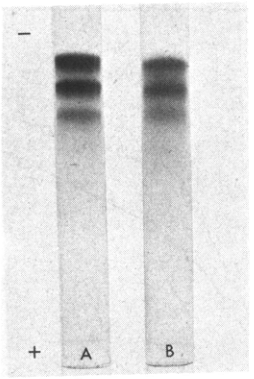

FIGURE 3: Polyacrylamide gel electrophoresis patterns of native globin (A) and globin(1-O_2) (B). Sample load is 50 μg. Bands migrated anodically.

continuum were observed, indicating that a significant amount of randomized photooxidation must have occurred.

An amino acid analysis of globin(1-O_2) was carried out to identify the amino acid(s) modified by photooxidation. Comparison of this analysis with one of native globin (Table I) clearly indicates that histidine is the only type of amino acid that is lost.[2] Only 1 histidine residue out of 12 is photooxidized, which is consistent with previous results (Breslow *et al.*, 1967) and with the fact that proto-sensitized photooxidation of free histidine requires approximately 1 mol of O_2/mol of amino acid (Mauk M. R., and Girotti, A. W., unpublished observation).

The fact that one histidine residue is destroyed in globin-(1-O_2) raises the question of whether or not this residue occupies a discrete position in the amino acid sequence. Our electrophoretic results (Figure 3) suggest that the photooxidation has occurred in a highly specific manner, possibly affecting only the susceptible residue(s) closest to the proto ring. We decided, therefore, to attempt to identify the sequence position of the residue(s) in question by degrading globin(1-O_2) proteolytically and examining the product for aberrant peptides by means of peptide mapping. A typical map of the soluble tryptic peptides of globin(1-O_2) is shown in Figure 4B. For comparison, a map prepared with an equal weight of soluble peptides from native globin is also shown (Figure 4A). Although the maps were prepared in identical fashion, one striking difference between them can be seen; ninhydrin spot 1 in the globin map is absent, or at best very faint, in the globin(1-O_2) map. Similarly, in maps sprayed with the Pauly reagent, Pauly-positive spot 1 was decidedly less intense in the case of photooxidized globin. We compared the color reactions of the peptide in spot 1 with those of two other peptides (circled in Figure 4A). Qualitatively spot 2 was found to behave similarly to spot 1 with ninhydrin and the Pauly reagent; complete disappearance of this spot, however, was noticed only after photooxidation to the 2-O_2 stage (not shown). To the eye, all other peptides remained unchanged, even after photooxidation to 2-O_2. Among these, the Pauly-reactive peptide in spot 3 was chosen as a typical unaffected peptide.[3]

The rapid disappearance of ninhydrin color in spot 1 indicates that the migration characteristics of peptide 1 have been altered. Based on the amino acid analysis of globin(1-O_2), the only possible change in this peptide is in its histidine content. We attempted to locate the photooxidized peptide on maps such as the one in Figure 4B, but could not make a positive identification because of the large number of background peptides. The relatively intense color of spot 1 (Figure 4A) suggested that this peptide might be recoverable from maps of native globin in amounts sufficient for amino acid analysis. Accordingly four maps were prepared and the areas of spot 1 were cut out and eluted free of peptide material. Only one peptide component was found when a sample from the eluate pool was subjected to high voltage paper electrophoresis at pH 1.7. The amino acid analysis of the recovered peptide is shown in Table II. On the basis of the known primary structure of sperm-whale myoglobin (Edmundson, 1965), peptide 1 is identified as the nonadecapeptide Lys^{78}-Lys-Gly-His-His-Glu-Ala-Glu-Leu-Lys-Pro-Leu-Ala-Gln-Ser-His-Ala-Thr-Lys^{96}. This peptide (designated as tryptic peptide 12 by Edmundson and Hirs (1962)) is distinctive in that it contains His^{93}, the imidazole group of which is known to coordinate directly with heme iron in crystalline myoglobin (Kendrew, 1962). A similar positioning of His^{93} and porphyrin ring in the proto–globin complex would be expected to promote a reaction at this histidine during proto-sensitized photooxidation. This could explain the rapid disappearance of peptide 1 during photooxidation.

The possibility that the disappearance of peptide 1 results from the photooxidation of His^{81} or His^{82} rather and His^{93} exclusively does not seem likely, since the first two are relatively distant from the porphyrin ring in crystalline myoglobin (Dickerson and Geis, 1969). This conclusion is supported

TABLE I: Amino Acid Composition of Native and Photooxidized Globin.

Amino Acid	Residues/Molecule		
	Native Globin[a]	Expected[b]	Globin-(1-O_2)[a]
Lysine	20.17	19	19.95
Histidine	12.13	12	10.87
Arginine	4.09	4	3.83
Aspartic acid	8.31	8	8.10
Threonine	4.85	5	4.94
Serine	5.44	6	5.87
Glutamic acid	18.62	19	19.07
Proline	4.46	4	4.13
Glycine	11.13	11	11.06
Alanine	17.00	17	17.00
Valine	7.42	8	7.08
Methionine	1.62	2	2.00
Isoleucine	7.48	9	7.57
Leucine	17.28	18	17.35
Tyrosine	2.99	3	2.88
Phenylalanine	5.83	6	5.86
Tryptophan[c]	2.12	2	2.13

[a] Based on alanine = 17.0. [b] Based on composition reported by Edmundson (1965). [c] Determined spectrophotometrically according to Edelhoch (1967).

[2] The possibility exists, however, that methionine is also photooxidized, but that this goes undetected because the resulting sulfoxide is converted back to methionine during acid hydrolysis (Ray and Koshland, 1960). This seems unlikely, however, in view of the observed oxygen uptake by histidine.

[3] This peptide was subsequently found to be unusually resistant to photooxidative modification; very little, if any, change in its ninhydrin or Pauly color could be detected after one reaction in which 12 O_2/mol of globin had been consumed (Mauk, M. R., and Girotti, A. W., unpublished observation).

TABLE II: Amino Acid Composition of Isolated Tryptic Peptides.

Amino Acid	Spot 1[a] Found[b]	Spot 1[a] Expected 78–96	Spot 2[a] Found[c]	Spot 2[a] Expected 90–96	Spot 3[a] Found[d]	Spot 3[a] Expected 35–42
Lysine	3.71	4	1.55	1	1.73	1
Histidine	2.60	3	1.05	1	1.00	1
Arginine					0.50	
Aspartic acid	0.16		0.26		0.61	
Threonine	0.98	1	0.85	1	1.18	1
Serine	1.12	1	1.16	1	0.86	1
Glutamic acid	3.02	3	1.00	1	2.37	2
Proline	0.80	1			1.16	1
Glycine	1.16	1	0.61		0.14	
Alanine	3.00	3	1.60	2	0.16	
Leucine	2.05	2	0.21		1.21	1
Phenylalanine					0.51	

[a] From Figure 4A. [b] Based on alanine = 3.0. [c] Based on glutamic acid = 1.0. [d] Based on histidine = 1.0.

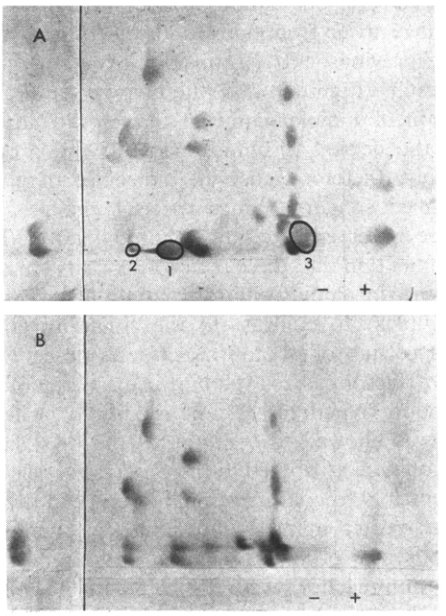

FIGURE 4: Peptide maps of the soluble tryptic peptides (3.1 mg) of native globin (A) and globin (1-O_2) (B). Ninhydrin positive spots are shown. The outlined spots (also Pauly positive) are discussed in the text.

by the identification of a second rapidly disappearing peptide (spot 2, Figure 4A). This peptide was isolated in the same way as peptide 1, and also characterized by amino acid analysis (Table II). From the major amino acid components, peptide 2 was identified as the heptapeptide Ala[90]-Gln-Ser-His-Ala-Thr-Lys[96]. This assignment is unequivocal despite the anomalous content of certain amino acids, *e.g.*, Lys and Gly. The latter may have been derived from contaminating peptide material, or from traces of amino acids eluted from the chromatography paper itself. Peptide 2 corresponds to F5-FG2 described by Banaszak *et al.* (1963) and must have arisen from an aberrant cleavage after Leu[89]. Since this peptide, a homolog of peptide 1, contains only one histidyl residue, His[93], there can be little doubt that this residue is selectively modified during limited photooxidation of proto–globin.

The amino acid composition of a photooxidation-resistant peptide, the one isolated from spot 3 (Figure 4A), is shown in Table II. This material appeared to be homogeneous on the basis of electrophoresis at pH 1.7, but amino acid analysis indicates the presence of more than one peptide. The major component clearly represents the octapeptide Ser[35]-His-Pro-Glu-Thr-Leu-Glu-Lys[42], which corresponds to tryptic peptide 20 of Edmundson and Hirs (1962). The presence of equimolar amounts of Asp, Arg, and Phe can be accounted for by the sequence Phe[43]-Asp-Arg[45]. From this it appears that spot 3 consists of at least two peptides, the octapeptide above, and its homolog which extends to Arg[45]. The latter must have originated from incomplete tryptic hydrolysis of the Lys[42]–Phe[43] bond, although the reason for this is not immediately obvious. The behavior of peptide 3 must reflect the relatively slow rate of photooxidation of His[36]. The structural implications of this observation will be discussed below.

One other peptide deserves some comment. It is not detectable on the map shown in Figure 4A and is presumably obscured by the seam in the paper. This peptide did appear as a faint ninhydrin and Pauly spot on subsequent maps in which the electrophoresis time was reduced from 100 to 85 min. It migrated between peptide 2 and the most cationic peptide (Figure 4A) and was tentatively identified as His[97]-Lys[98], on the basis of information provided by Banaszak *et al.* (1963). This identification proved to be correct, since synthetic His-Lys was found to migrate to the same position on a peptide map. His[97] is noteworthy since its imidazole group appears to interact with a propionate side chain of heme in crystalline myoglobin (Banaszak *et al.*, 1963). On the basis of proximity alone, one might expect that the photooxidation of His[97] would be favored if its association with the porphyrin ring persisted in the proto–globin complex. Unlike peptide 1, His[97]-Lys[98] was found in very low yield; on some peptide maps it was partially obscured by background color after spraying with ninhydrin or Pauly's reagent. Consequently, from the maps of native globin and globin(1-O_2) examined thus far, no definite conclusion about the photoreactivity of His[97] can be reached.

Discussion

Certain differences between the photooxidation conditions described in this report and those cited previously (Breslow *et al.*, 1967) should be discussed. From spectral data, Breslow *et al.* (1967) deduced that the 1:1 complex of protoporphyrin IX and apomyoglobin dissociates in the light, and that free proto might then act as a nonspecific photosensitizer of the protein. In an attempt to avoid this problem, they carried out photooxidation reactions with high globin : proto molar ratios, *i.e.*, tenfold or greater. It was reasoned that any released proto could reassociate with excess native globin before the onset of randomized photooxidation, and the reaction could be stopped when all the native globin had been consumed. As pointed out by Breslow *et al.* (1967), however, this approach would be futile if the rate of photosensitization by free proto were significantly greater than the rate of complex formation. Because of this possible complication, we decided to work directly with the equimolar mixture of proto and globin, and

to examine the photooxidation product for any heterogeneity that may have arisen from nonspecific reactions. This had not been done previously. It is apparent from our gel electrophoresis results (Figure 3) that photooxidation of globin to a consumption of 1 molar equiv of oxygen did not alter significantly the degree of homogeneity of the protein. This would imply, therefore, that all molecules of globin(1-O_2) were modified similarly. Since spectral changes in proto–globin were observed after this photooxidation (Figure 1), it is our contention that these reflect minor structural changes in the porphyrin binding site rather than actual dissociation of the complex. In contrast to the situation with globin-(1-O_2), a spectrum of photoproducts was observed in more extensively photooxodized globin, *e.g.*, globin(4-O_2). Evidently random reactions became significant when photooxidation was allowed to continue to the 4-O_2 stage. Such reactions could be attributed to the progressive release of free proto or possibly to conformational changes in the complex, exposing a greater number of residues to random attack. Consequently, in order to achieve maximum selectivity, photooxidation reactions were stopped after the consumption of no more than 1 or 2 molar equiv of oxygen.

Our studies have indicated that one particular residue in proto–globin, His^{93}, is highly susceptible to photooxidation. This finding is significant, in view of established evidence for the three-dimensional structure of myoglobin. As pointed out by Kendrew (1962), an imidazole nitrogen of His^{93} is coordinated directly with heme iron in crystalline myoglobin. It is apparent from our results that the close positioning of His^{93} and the porphyrin ring must persist in the proto–globin complex, despite the absence of heme iron. This is consistent with reported physicochemical evidence that the overall conformation of proto–globin in solution is similar to that of metMb (Breslow *et al.*, 1967). It is interesting to note that Banaszak *et al.* (1963), using an entirely different experimental approach from ours, were able to obtain comparable information relating to the structure of the porphyrin binding site in myoglobin. On treating metMb with bromoacetate, they found that His^{93} was completely resistant to carboxymethylation, presumably because of its interaction with protoheme.

In contrast to His^{93}, His^{36} was found to be very insensitive to photooxidation. In the three-dimensional structure of myoglobin at the 2-Å level, His^{36} appears to lie in the general vicinity of the heme group, but does not interact with it (Kendrew, 1962). Instead, its imidazole ring makes contact with the phenyl ring of the Phe^{106} (Banaszak *et al.*, 1963). The lack of reactivity of His^{36} toward bromoacetate (Banaszak *et. al.*, 1963) has been attributed to the interaction of this residue and Phe^{106}. This same interaction, occurring in the proto–globin complex, may also account for the low photooxidizability of His^{36}. Therefore, the determining factor in the rate of photooxidation of a particular side chain in proto–globin may not necessarily be the degree of spatial separation of the side chain and the photosensitizer. Certain facets of the native protein structure, such as the one described above, may exert a controlling influence.

It should be pointed out that only the soluble tryptic peptides of native and photooxidized globin were analyzed by peptide mapping (Figure 4A and B). These represent about 70% by weight of the globin molecule. The remaining peptides, which constitute the so-called "core" fraction, were insoluble at pH 6.4, and impossible to separate by the mapping technique described. According to Edmundson (1965), the insoluble fraction consists of three different peptides, one of which contains His^{64} in the globin sequence. In crystalline myoglobin this residue lies close to the heme ring, and on the opposite side of the iron-linked residue, His^{93} (Kendrew, 1962). There is a distinct possibility, therefore, that His^{64} in the proto–globin complex will resemble His^{93} in being highly sensitive to photooxidation. We are currently seeking experimental means of investigating this possibility.

On the basis of recent developments (Spikes and MacKnight, 1970), it is evident that dye-sensitized photooxidation, when properly carried out, can serve as a powerful tool for examining protein structure in solution. A key factor in the success of this technique has been the ability to achieve selective modification of photooxidizable groups. Certain photosensitizers have been used to distinguish between "exposed" and "buried" residues in various proteins (Kenkare and Richards, 1966; Jori *et al.*, 1970b). Other sensitizers, either covalently attached or bound with high affinity to specific sites of interest, have provided important structural information about such sites (Rippa and Pontremoli, 1969; Jori *et al.*, 1970a; Coulson and Yonetani, 1972). Many of these investigations have been carried out initially on model proteins, the conformations of which have been elucidated by other physical or chemical means. Such an approach has been taken in the present study. We have presented evidence that protoporphyrin IX, when bound to apomyoglobin, can bring about the selective photooxidation of at least one side chain in its immediate binding environment. In principle, the technique described for myoglobin should be applicable to other hemoproteins in which protohemin can be replaced by proto. Accordingly, proto-sensitized photooxidation may prove to be a valuable means of probing the heme binding sites of hemoproteins whose structures have not been well characterized.

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Distinct Effects of Diamines, Polyamines, and Magnesium Ions on the Stability of λ Phage Heads†

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ABSTRACT: The DNA condensed in a tailless λ phage head is readily lost from the structure and appears free in solution. These heads provide a model system for studying interactions in a condensed DNA structure. The diamine, putrescine, stabilizes heads and maintains them in a biologically functional condition but the polyamines, spermidine and spermine, as well as the divalent metal ions, Mg^{2+} and Ca^{2+}, destabilize heads. Putrescine eliminates or moderates the sensitivity of heads to these destabilizing agents. Thus, if the DNA is to remain condensed, the cations that neutralize the DNA phosphate anionic groups also must possess the appropriate steric properties.

Synthesis of the phage head during λ morphogenesis is not dependent upon concomitant synthesis of the tail. Mutants defective in a tail gene produce a full titer of normal heads (Weigle, 1966; Harrison *et al.*, 1973). The stability of these tailless heads differs from that of complete phage. This report describes conditions under which DNA is retained or lost from a head. Of particular interest is the interplay of diamines, polyamines, and metal ions with the phage proteins and DNA. From a description of how they alter stability of the condensed state of λDNA in the phage head, we hope to define parameters that are important for stabilizing the close packing of any DNA.

Materials and Methods

Media and Buffers. K medium is prepared by autoclaving 1 g of NH_4Cl, 6 g of Na_2HPO_4, 3 g of KH_2PO_4, and 1 g of NaCl in 850 ml of glass-distilled water, cooling, and then adding from sterile stock solutions 2 ml of 1 M $MgSO_4$, 10 ml of 2.5×10^{-2} M $CaCl_2$, and 150 ml of 10% Norit A treated Casamino acids. TPBE contains 10^{-2} M Tris-HCl (pH 7.1), 10^{-2} M putrescine-HCl, 10 mg/ml of bovine serum albumin, and 2×10^{-4} M EDTA. TBE has the same composition as TPBE except that putrescine is omitted. Tris–EDTA is 10^{-2} M Tris-HCl with 10^{-3} M NaEDTA, pH 7.1.

Putrescine dihydrochloride, cadaverine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, and all the amino acids except hydroxylysine were purchased from Sigma Chemical Co. and used without further purification. The basic amino acids were obtained as the hydrochlorides and, unless it is otherwise specified, all solutions were tested for their effect on head stability at a pH near neutrality.

Purification of λ Heads. A culture of W3350 *su*⁻ (λ*sus Jam*27) was grown in K medium at 37° to 5×10^8 cells/ml. The cells were chilled, induced with ultraviolet light, and then returned to the shaker bath until the OD at 590 nm indicated lysis was complete. (When preparing heads with ^{3}H-labeled DNA, 1 mCi of [*methyl*-^{3}H]thymidine was added per liter of culutre at 20 min after induction.) After chilling, 1 ml of $CHCl_3$ was added and the debris removed by centrifugation for 45 min at 10,000*g*. In the text, the lysate at this stage of purity is referred to as crude heads. Purified heads were obtained as described by Harrison *et al.* (1973).

Preparation of λ Tails. The source of tails in these experiments was W3350 *su*⁻ (λ*Aam*32 *Bam*1) grown in K medium and induced as described for heads. After the addition of $CHCl_3$ and the removal of cell debris by centrifugation, lysates are stored at 4°. Crude lysates contain 8×10^{10}–2×10^{11} tails/ml and the tails remain active for months. Pure tails are obtained as described elsewhere (Harrison *et al.*, 1973).

In Vitro Head–Tail Joining. Heads are titered by mixing 0.1

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