Protoporphyrin-Sensitized Photodamage in Isolated Membranes of Human Erythrocytes†

Albert W. Girotti

ABSTRACT: Protoporphyrin IX (proto) is a potent photosensitizer of membrane damage in human erythrocyte ghosts. Under aerobic conditions (pH 8, 10 °C), exposure of ghosts in the presence of proto to filtered blue light resulted in the formation of large (>250 000 daltons) aggregates of membrane proteins, as visualized by sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis. Correspondingly, there was a rapid disappearance of polypeptide bands 1, 2, 4.1, and 6 and a slower decay of the remaining bands. Postirradiation treatment of membranes with dithiothreitol or pretreatment with *N*-ethylmaleimide had little effect on aggregation, indicating that intermolecular –S–S– bonds (exclusive of other bonds) are not produced to any appreciable extent in the photoreaction. Photoaggregates isolated by $NaDodSO_4$–Sepharose chromatography were resistant to dissociation in boiling 1% $NaDodSO_4$–8 M urea, which is strong supporting evidence that stable cross-links are produced. Amino acid analysis of total protein from extensively cross-linked membranes revealed significant losses of histidine, cysteine, tyrosine, and tryptophan, raising the possibility that some of these residues participate in cross-linking. The antioxidants butylated hydroxytoluene and *N,N'*-diphenyl-*p*-phenylenediamine were effective inhibitors of lipid peroxidation (as measured by malonaldehyde formation) in irradiated membranes. However, protein cross-linking, photoinactivation of Na^+,K^+-ATPase and acetylcholinesterase, and loss of SH groups were unaffected by these agents, suggesting that lipid peroxidation does not mediate the observed protein damage. It follows that malonaldehyde, originating as a byproduct of peroxidation, probably plays no role in protein cross-linking. As further support for this conclusion, membranes in which cross-linking was induced by exogenous malonaldehyde were shown to be strongly fluorescent, with an emission maximum at 460 nm (excitation at 390 nm), whereas photooxidized membranes had no fluorescence in the 400–600-nm region.

Red blood cells of individuals with erythropoietic protoporphyria contain abnormally large amounts of protoporphyrin IX, a potent photosensitizing agent. These cells, as well as normal ones to which proto[1] is added, readily photohemolyze in vitro when irradiated with visible light in the presence of O_2 (Fleischer et al., 1966; Schothorst et al., 1970; Hsu et al., 1971). Hemolysis stems from membrane damage with ensuing cation leaks and colloid osmotic swelling. This effect can be looked upon as a simple analogue of cutaneous photodamage that often occurs in protoporphyrics. In recent years a number of investigators have attempted to characterize at the molecular level the membrane lesions that are ultimately responsible for lysis. Since irradiated porphyric red cells undergo peroxidative degradation of polyunsaturated membrane lipids, it has been postulated (Goldstein & Harber, 1972) that hemolysis is either due to peroxidation per se or to secondary effects triggered by this free-radical process. Previous studies in this laboratory (Girotti, 1976a) and elsewhere (De Goeij et al., 1975, 1976) have shown that photoinduced lipid peroxidation in proto-containing red cell membranes is accompanied by polypeptide aggregation, as seen by $NaDodSO_4$ gel electrophoresis. Such aggregation, which was presumed to be due to intermolecular cross-linking, could conceivably be a damaging side effect of lipid peroxidation. One of the major objectives of the present work was to examine this question in an effort to better understand the photoaggregative process.

Experimental Section

Materials. Protoporphyrin IX dimethyl ester, ultrapure urea, and ultrapure guanidine hydrochloride were obtained from Schwarz/Mann. Proto was prepared from its ester as described previously (Mauk & Girotti, 1973). Trypsin (TRTPCK) was purchased from Worthington Biochemical Corp. Sepharose 4B, butylated hydroxytoluene, 5,5'-dithiobis(2-nitrobenzoic acid), acetylthiocholine chloride, D,L-glyceraldehyde 3-phosphate diethyl acetal (Ba^{2+}), NAD^+, NADH, ATP, ouabain, lactate dehydrogenase (type II from rabbit muscle), pyruvate kinase (type II from rabbit muscle), and phosphoenolpyruvate were obtained from Sigma. Dithiothreitol, 2-thiobarbituric acid, and 1,1,3,3-tetramethoxypropane were Aldrich products, while *N,N'*-diphenyl-*p*-phenylenediamine and the materials used for $NaDodSO_4$ gel electrophoresis were from Eastman. All other chemicals were of reagent quality, and all solutions were prepared with glass-redistilled water.

Preparation of Membranes and Membrane Extracts. Freshly drawn human blood in citrate–phosphate–dextrose solution was obtained from the Milwaukee Blood Center and used within a 2-week period. Unsealed erythrocyte membranes (ghosts) were prepared as described previously (Girotti, 1975). The lysing and washing buffer was either 6 mM sodium phosphate (pH 8.0 at 4 °C) or 12 mM Tris-HCl (pH 8.0 at 4 °C), the latter being used exclusively for all preparations in which ATPase activity would be measured or lipid phosphate determined. Membrane suspensions were standardized on the basis of total protein (Lowry et al., 1951) after solubilization in 0.2% $NaDodSO_4$; bovine serum albumin was used as the protein standard.

Extraction of spectrin (bands 1 and 2) from ghosts was carried out essentially as described by Fairbanks et al. (1971). Stock membranes in 6P8 were diluted with 15 volumes of 0.1 mM EDTA and incubated for 15 min at 37 °C. Following

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[1] Abbreviations used: proto, protoporphyrin IX; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, (ethylenedinitrilo)tetraacetic acid; 6P8, 6 mM sodium phosphate buffer (pH 8.0); 30P8, 30 mM sodium phosphate buffer (pH 8.0); MDA, malonaldehyde; BHT, butylated hydroxytoluene (2,6-di-*tert*-butyl-4-methylphenol); DPPD, *N,N'*-diphenyl-*p*-phenylenediamine.

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centrifugation, the supernatant solution (containing spectrin and traces of band 5 protein) was dialyzed exhaustively at 4 °C against a pH 7.7 buffer containing 50 mM sodium phosphate, 0.1 M NaCl, 0.1 mM EDTA, an 2 mM β-mercaptoethanol. Immediately before irradiation, the spectrin solution was concentrated approximately 15-fold by means of a hollow fiber device (Bio-Rad Laboratories) and dialyzed against 50 mM sodium phosphate and 0.1 mM EDTA (pH 7.8). When needed for irradiation experiments, membrane vesicles depleted of spectrin and band 5 were recovered and washed extensively with 6P8.

Irradiation. Irradiation reactions were carried out at 10 ± 1 °C in a thermostated stirrer bath (Yellow Springs Instruments, Model 5301) equipped with a Plexiglas top and sides. As many as four reactions could be carried out simultaneously in this apparatus with constant magnetic stirring. Aerobic conditions were used, except where indicated. Illumination was performed with a 250-W Duro-Test lamp (Model R40) positioned above the stirrer. Incident light was restricted to the Soret band absorption region of proto by placing a blue filter (Corning no. 5030; transmission maximum ~420 nm) between the lamp and the reaction chamber. Uniform light exposure was achieved by enclosing the chamber in a cylindrical metal reflector. Light intensity near the sample surface was measured with a radiometer (Yellow Springs Instruments, Model 65A) and maintained at ~200 W/m^2 unless indicated otherwise. Volumes of samples to be illuminated were kept as small as possible to ensure narrow light paths (usually <5 mm).

Enzyme Assays. Samples of irradiated ghosts which were drawn for enzyme activity determinations were stored on ice in the dark and used within 24 h. Previous studies (Girotti, 1976b) showed that the photosensitized inactivation of membrane-bound Na^+,K^+-ATPase, acetylcholinesterase, and glyceraldehyde-3-phosphate dehydrogenase is strictly light dependent, and no additional activity loss (relative to unirradiated controls) occurs during subsequent dark periods up to 24 h.

ATPase activity was measured by coupled enzymatic assay, using the auxiliary enzymes pyruvate kinase and lactate dehydrogenase. Reaction mixtures (1.5 mL) contained the following components: 50 mM Tris-HCl (pH 7.4 at 44 °C), 0.3 mg of membrane protein, 2.5 mM ATP, 0.38 mM phosphoenolpyruvate, 0.38 mM NADH, 2.3 units of pyruvate kinase, and 4.1 units of lactate dehydrogenase; for total ATPase activity (Mg^{2+}-ATPase + Na^+,K^+-ATPase), 88 mM NaCl, 35 mM KCl, and 5 mM $MgCl_2$ were included; for Mg^{2+}-ATPase alone, an identical reaction mixture was prepared except for the inclusion of 0.5 mM ouabain. Blanks containing heat-denatured ATPases were prepared alongside. Reactions were started by the addition of ATP, allowed to run for 1 h at 44 ± 1 °C, and terminated by the addition of 10 mM EDTA. The samples were centrifuged at 20000*g* for 15 min, and the absorbance of the supernatant solutions was measured at 340 nm.

Glyceraldehyde-3-phosphate dehydrogenase and acetylcholinesterase were determined according to Steck & Kant (1973), with minor variations in procedure (Girotti, 1976b).

Sulfhydryl Determinations. The SH content of photooxidized ghosts was determined by titration with 5,5′-dithiobis(2-nitrobenzoate), as outlined previously (Girotti, 1978). Titrations were carried out in the presence of 1% $NaDodSO_4$ to expose buried SH groups.

Lipid Peroxidation. Peroxidation of polyunsaturated lipids in irradiated ghosts was monitored by means of the thiobarbituric acid assay. The principal reactant with thiobarbituric acid in this assay is malonaldehyde, a breakdown product of peroxides derived from fatty acyl groups with three or more double bonds (Dahle et al., 1962; Buege & Aust, 1978). The procedure used in the present work was adapted from that of Shimasaki & Privett (1975). All steps were carried out under minimal illumination. The membrane sample (0.5 mL, containing 1.0 mg of membrane protein) was mixed with 1.0 mL of cold 10% trichloroacetic acid. After being incubated for 5 min on ice, the suspension was centrifuged and 1.0 mL of the supernatant solution was mixed with an equal volume of 0.67% 2-thiobarbituric acid and 1 mM EDTA. [EDTA was included to inhibit possible Fe^{3+} enhancement of the color yield (Wills, 1964).] After being heated for 10 min in a boiling water bath, the sample was cooled and its absorbance at 535 nm was recorded. All absorbance readings were corrected for values given by reagent blanks (lacking membranes) and appropriate membrane blanks (lacking thiobarbituric acid). The latter readings accounted for any contribution of proto to A_{535}. Absorbance values were converted to malonaldehyde concentrations by using an extinction coefficient of 155 mM^{-1} cm^{-1}; this was determined by reacting thiobarbituric acid with known concentrations of malonaldehyde generated from 1,1,3,3-tetramethoxypropane (Girotti, 1975).

Electrophoresis. Conventional $NaDodSO_4$–polyacrylamide gel electrophoresis of ghost proteins was carried out according to established procedures (Fairbanks et al., 1971; Steck & Yu, 1973). Electrophoresis on composite gels of 3.5% polyacrylamide and 0.4% agarose (Steck, 1972) was also employed, primarily to improve the resolution of photoaggregated polypeptides. Unless indicated otherwise, membrane samples to be electrophoresed were solubilized with 1% $NaDodSO_4$ and incubated in the presence of 200 mM dithiothreitol for 30 min at 37 °C. Staining was accomplished with Coomassie blue R-250. Densitometric scanning of stained gels was carried out at 550 nm with a Gilford linear transport accessory. Molecular weights of protein photoaggregates were estimated by using polypeptides 1–7 as standards (Steck, 1972).

Amino Acid Analyses. These were carried out on the total population of ghost proteins. The procedure for extracting proteins in an essentially lipid-free state was adapted from that of Rega et al. (1967). Both irradiated ghosts and unirradiated controls were treated as follows under minimal room lighting. A sample of membranes (2 mg of protein/mL in 6P8) was washed twice with 2.5 volumes of cold H_2O; the supernatant fractions (containing spectrin primarily) were pooled and dialyzed vs. H_2O. This step reduced the ionic strength of the membrane suspension to below 0.5 mM, thereby allowing more efficient protein extraction (Rega et al., 1967). The suspensions were then adjusted to approximately pH 8.8 with 0.1 N NaOH and subjected to tryptic hydrolysis. Two aliquots of trypsin (totaling 2.5% w/w of the membrane protein) were added, with 1 h of incubation at 25 °C after each addition. Following hydrolysis, the membranes were chilled, mixed with 0.5 volume of cold butanol, and vortexed vigorously for 1 min. After being allowed to stand for 15 min on ice, the suspension was centrifuged (18000*g* for 15 min) and the aqueous phase was removed. The extraction was repeated by mixing the butanol layer with 2 volumes of H_2O. The combined aqueous extracts (including the initial washes that were dialyzed) were lyophilized, and the recovered protein was stored over P_2O_5 at −20 °C.

Gel electrophoresis revealed that tryptic digestion, as described above, completely reduced photoaggregated proteins

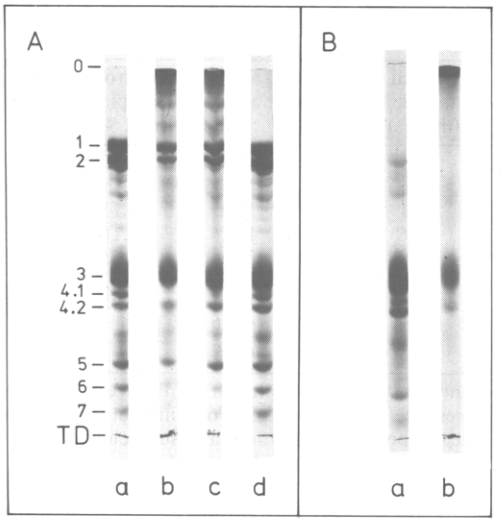

FIGURE 1: Protoporphyrin-sensitized photoaggregation of membrane proteins. (A) Ghosts (2.0 mg of protein/mL in 6P8) were irradiated for 20 min (light intensity ~350 W/m^2) in the presence of 0.5 μM proto and then subjected to polyacrylamide–agarose/NaDodSO$_4$ gel electrophoresis without prior reduction (gel b). A duplicate sample was treated with 200 mM dithiothreitol before electrophoresing (gel c). Gel a is a dark control containing proto, and gel d is an irradiated control lacking the porphyrin. Protein load, 36 μg/gel; TD, tracking dye (bromphenol blue). (B) Ghosts depleted of bands 1, 2, and 5 were irradiated as described above (gel b). Gel a is the dark control. Protein load, 44 μg/gel.

to low molecular weight fragments, thereby greatly improving extractability. Without trypsinization, protein recovery [determined by the method of Lowry et al. (1951)] was quantitative in native ghosts but only ~75% in ghosts irradiated for 1 h in the presence of 1 μM proto. After trypsin treatment, >95% of the protein in the photooxidized sample was extractable.

Analyses of amino acids other than tryptophan and cysteine were performed on lyophilized protein samples that had been hydrolyzed in 6 N HCl by heating at 110 °C for 24, 32, and 48 h. A Beckman, Model 120B, analyzer was employed. Cysteine content was obtained by determining SH groups in whole membranes, as described above. Tryptophan was determined in totally extracted membrane protein by the spectrophotometric method of Edelhoch (1967). The solubilizing medium was 6 M guanidine hydrochloride and 20 mM sodium phosphate (pH 6.5); corrections were made for small amounts of light scattering (Donovan, 1969).

Fluorescence Measurements. Cross-linking of ghost proteins by malonaldehyde was carried out essentially as described previously (Girotti, 1975). After solubilization of the cross-linked membranes in 3% NaDodSO$_4$–10 mM dithiothreitol, fluorescence spectra were recorded with an Aminco SPF-125 Fluorimeter. Ghosts in which photosensitized cross-linking had been produced were analyzed similarly.

Results

Effect of Photooxidation on Membrane Proteins. When erythrocyte ghosts are irradiated in the presence of submicromolar concentrations of proto, striking changes are seen in the NaDodSO$_4$ gel electrophoretic profile of membrane proteins. Results of a typical experiment are shown in Figure 1A. Exposure of the membranes to 0.5 μM proto and a light dose of ~40 J/cm^2 caused a pronounced diminution in staining intensity of polypeptide bands 1, 2, 4.1, 6, and possibly 7, with a corresponding appearance of large aggregates near the origin (gel b). Following the time course of photoaggregation consistently revealed a rapid disappearance of the above bands relative to bands 3, 4.2, 5, and the sialoglycoproteins (not shown). The polyacrylamide–agarose gels used allowed a partial resolution of the photoaggregates into constituents ranging in molecular weight from ~320 000 (the band immediately above band 1) to ~450 000. No effect was seen in a dark control (gel a) or in an irradiated control lacking proto (gel d). Photoaggregation was substantially reduced when irradiation was carried out under O_2 depletion (e.g., in an argon atmosphere), suggesting that the reaction is photodynamic in nature (Spikes & MacKnight, 1970).

Photosensitized oxidation of free cysteine to cystine has been observed (Gennari et al., 1974). It was of interest, therefore, to determine whether intermolecular –S–S– cross-linking plays any role in the effect described here. Accordingly, a duplicate of sample b in Figure 1A was incubated with a high concentration of dithiothreitol (0.2 M) prior to electrophoresis (gel c). A comparison of gels c and b reveals only a small intensification of spectrin after reductive treatment, indicating that –S–S– bond formation (exclusive of other types of bonding) accounts for relatively little of the observed aggregation. This does not necessarily imply that SH groups are not properly juxtaposed for cross-linking, since such reactions have been shown to occur via other oxidative mechanisms, e.g., catalysis by the Cu^{2+}(*o*-phenanthroline)$_2$ complex (Steck, 1972).

On a mass basis, spectrin is the most abundant of the red cell membrane proteins, with the exception of band 3. Therefore, one would expect photoaggregates such as those seen in Figure 1A to be composed predominantly of spectrin. This assumption was checked by irradiating ghosts that had been depleted of bands 1, 2, and 5 (Fairbanks et al., 1971). When such ghosts [which were actually in the form of membrane vesicles (Steck & Yu, 1973)] were photooxidized under the same conditions as whole ghosts (Figure 1A), the results shown in Figure 1B were obtained. A small amount of material is still seen near the origin (gel b), indicating that removal of bands 1, 2, and 5 does not preclude photoaggregation of the remaining peptides. On the other hand, the intermediate bands (300 000–450 000 daltons) observed with whole ghosts are absent, suggesting that spectrin and possibly band 5 are constituents of these bands.

It was of interest to learn whether participation of spectrin in photoaggregation depends on its association with the membrane. As shown in Figure 2A, exposure of isolated spectrin to a light dose of ~85 J/cm^2 in the presence of 1 μM proto resulted in a 20% loss of bands 1 and 2 and the appearance of at least two new bands, a (~400 000 daltons) and b (~480 000 daltons). The results of a companion experiment with ghosts containing the same concentration of bound spectrin are shown in Figure 2B. In this case, diminution of bands 1 and 2 after irradiation amounted to ~85%; the photoaggregates produced were much larger and barely entered the gel. It is clear from these experiments that although solubilized spectrin is still susceptible to photosensitized aggregation, the extent of the reaction under a given set of conditions is much less than that with membrane-bound spectrin. A likely explanation for these results is that the bound protein exists in a tight, meshlike array on the inner membrane surface, whereas the free protein is primarily a dimer (band 1 + 2) and undergoes fewer protein–protein contacts (Ralston et al., 1977).

It is conceivable that the photoproducts described are not aggregates, as indicated, but individual polypeptides that happen to migrate more slowly after extensive photooxidation. Such an effect could be attributed to diminished NaDodSO$_4$

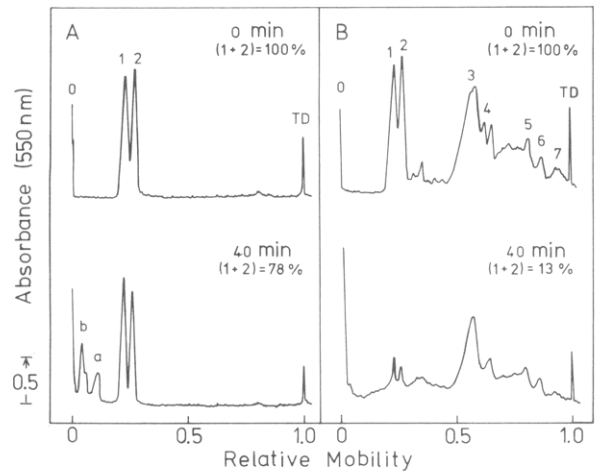

FIGURE 2: Photoaggregation of spectrin in solubilized and membrane-bound forms. Densitometric scans of polyacrylamide–agarose/$NaDodSO_4$ gels showing (A) isolated spectrin [0.5 mg/mL in 50 mM sodium phosphate and 0.1 mM EDTA (pH 7.8)] and (B) whole membranes [2.0 mg of protein/mL in 50 mM sodium phosphate (pH 7.8)] before and after 40 min of irradiation in the presence of 1.1 μM proto. The preparations were irradiated simultaneously (light intensity $\sim$350 W/m^2). Protein load: spectrin, 15 μg/gel; membranes, 61 μg/gel. Values in parentheses represent relative integrated areas under bands 1 and 2.

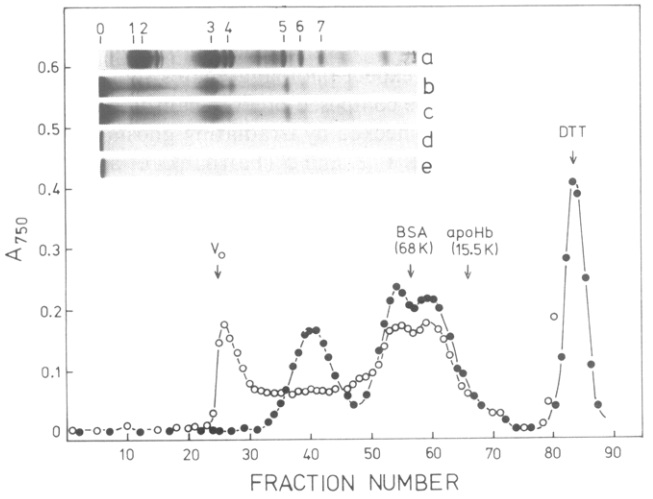

FIGURE 3: Chromatographic profile of membrane proteins before and after photooxidation. Ghosts (2.9 mg of protein/mL in 30P8) were exposed to a light dose of $\sim$85 J/cm^2 in the presence of 1 μM proto. A sample (5.0 mg of protein) was solubilized in 3% $NaDodSO_4$ and 10 mM dithiothreitol, heated for 2 min at 100 °C, and applied to a Sepharose 4B column (1.5 × 40 cm) that had been equilibrated with a pH 8.1 buffer consisting of 25 mM Tris-HCl, 0.1 mM EDTA, and 1% $NaDodSO_4$. Elution was carried out with this buffer at 25 °C (flow rate 6 mL/h; fraction volume 1 mL). Aliquots of 0.3 mL from each fraction were assayed for protein by the Lowry method (Lowry et al., 1951), in which absorbance at 750 nm was measured (○). An unirradiated control was prepared and chromatographed similarly (●). The column was calibrated with blue dextran 2000 (V_0, void volume), the reduced monomer of bovine serum albumin (BSA, 68 000 daltons), and the subunit of apohemoglobin (apoHb, 15 500 daltons). The peak between fractions 80 and 90 is due to dithiothreitol. The inset shows $NaDodSO_4$ gels of the control (a) and irradiated ghosts before (b) and after (c) heating for 2 min at 100 °C following solubilization (see Experimental Section). A sample from the pool of fractions 25–28 (○) is also shown before (d) and after (e) boiling for 2 min in 1% $NaDodSO_4$ and 8 M urea.

binding (Steck & Fox, 1972). Therefore, as an alternative to $NaDodSO_4$ gel electrophoresis, Sepharose 4B chromatography in the presence of $NaDodSO_4$ was used to separate irradiated ghost proteins. Figure 3 shows a typical elution profile before and after photooxidation. The unirradiated control exhibits three overlapping protein peaks between fractions 30 and 70. The first of these (fractions 32–48) represents the spectrin subunits predominantly, while the second two (fractions 50–70) represent a mixture of bands 3–7. In the photooxidized sample, a prominent new peak is seen near the void volume; correspondingly, there is a large decrease in the spectrin peak and a somewhat smaller decrease in the remaining protein peaks. These changes broadly mimic those observed electrophoretically on the same samples (compare gels a and b in Figure 3). It should be noted that heating the irradiated membranes at 100 °C after solubilization did not reverse photoaggregation to any significant extent, judging from the similar staining intensities of spectrin in gels b and c. Fractions under the void volume peak of the photooxidized sample were pooled and examined electrophoretically (gel d). It is obvious that this material consisted entirely of photoaggregates, with no evidence of slow dissociation of constituent peptides. More importantly, the aggregates were stable under strong denaturing conditions, e.g., boiling in 1% $NaDodSO_4$ and 8 M urea (gel e). This is strong supporting evidence that cross-links of some type(s) are produced in the photoreaction rather than noncovalent interactions which happen to resist disruption by detergent alone [cf. Steck & Fox (1972)].

Table I: Amino Acid Composition of Membrane Protein before and after Photooxidation[a]

amino acid	control sample (mol %)	irradiated sample (mol %)	amino acid	control sample (mol %)	irradiated sample (mol %)
Lys	6.01	6.18	Ala	8.12	8.18
His	3.09	1.88	Val	7.15	6.95
Arg	4.81	4.79	Met	2.15	2.05
Asp	9.06	9.38	Ile	5.10	4.91
Thr	5.49	6.00	Leu	11.55	11.33
Ser	5.75	6.45	Tyr	2.62	2.22
Glu	13.40	13.35	Phe	3.90	3.86
Pro	4.37	4.66	Trp[b]	153	122
Gly	6.95	6.92	Cys[c]	74	43

[a] Membranes (2.0 mg of protein/mL in 6P8) were irradiated for 1 h in the presence of 1 μM proto. Total membrane protein was extracted (see Experimental Section) and subjected to amino acid analysis. An unirradiated control was analyzed alongside. Values shown are averages of 24-, 32-, and 48-h hydrolyses, except for serine and threonine, which were extrapolated to zero hydrolysis time. [b] Determined in protein extract by the method of Edelhoch (1967). Values are given in units of nmol/mg of protein. [c] Determined according to Ellman (1959) in membranes solubilized with 1% $NaDodSO_4$. Values are given in units of nmol/mg of protein.

Membrane proteins from a photooxidized preparation of ghosts were subjected to amino acid analysis to identify photolabile residues (Table I). Protein cross-linking in the sample shown was extensive and similar to that seen in Figure 3 (gel b). The amino acid composition of total extracted protein is compared with that of a nonirradiated control. It should be noted that the values obtained with the latter are in good agreement with those reported by Rosenberg & Guidotti (1968), who used a completely different method of protein extraction. As can be seen, the only residues that were significantly affected by irradiation are those known to be susceptible to photodynamic action (Spikes & MacKnight, 1970), viz., histidine, cysteine, tryptophan, and tyrosine.[2] The approximate losses of these residues under the conditions of

[2] Methionine is also photooxidizable. However, no conclusion can be reached about this amino acid since its expected photoproduct, methionine sulfoxide, reverts to the parent molecule during acid hydrolysis (Ray & Koshland, 1960).

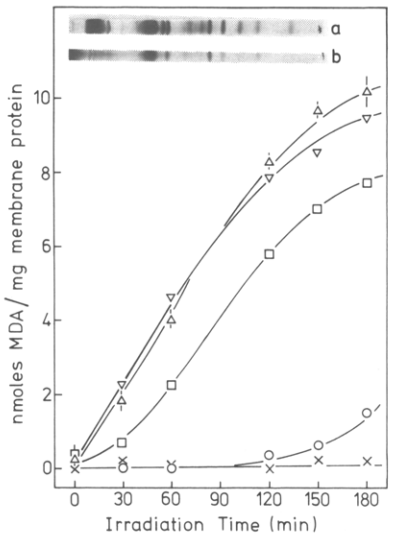

FIGURE 4: Protoporphyrin-sensitized lipid peroxidation in ghosts, as measured by malonaldehyde (MDA) formation. Membrane samples (2.0 mg of protein/mL in 30P8) were irradiated in the presence of the following concentrations of proto: 0 (×); 1 (○); 10 (□); 50 (△); and 100 μM (▽). The light intensity was 200 W/m^2. MDA values in the experiment using 50 μM proto are means ± deviation of duplicate determinations. The inset shows electropherograms of the sample containing 1 μM proto before (a) and after (b) 1 h of irradiation.

this experiment were 40, 42, 20, and 15%, respectively. The large decrease in cysteine content cannot be accounted for by intermolecular –S–S– bond formation, since, as already pointed out, relatively little of this is observed. Inasmuch as free cysteine is usually photooxidized to cysteic acid (Spikes & MacKnight, 1970), it can be assumed that the same is true in the present system. It is tempting to speculate that the disappearance of some of the above residues is at least partially attributable to cross-linking reactions. In the case of histidine and tyrosine, this presumes, of course, that any bonds that are formed are stable to acid hydrolysis. It should be recognized, however, that even if losses due to cross-linking were distinguishable from those due to other reactions, the former might well be smaller than the detection limits of the analyses used. [As an extreme example, cross-linking within the spectrin dimer (~2000 amino acids per subunit) might involve only two residues.] This could also hold true for any nonphotooxidizable residues, if they happen to participate in the reaction.

Lipid Peroxidation. It is well-known that lipid peroxidation in biomembranes is deleterious to membrane structure and function. Free-radical reactions associated with peroxidation are not only damaging to the lipid bilayer itself but may also produce secondary damage to membrane-bound enzymes and structural proteins (Tappel, 1975). It was of interest in the present study, therefore, to determine (a) whether proto can sensitize measurable peroxidation in irradiated ghosts and (b) if so whether any relationship exists between this effect and protein cross-linking. As shown in Figure 4, irradiation of ghosts in the presence of sufficient concentrations of proto resulted in peroxidation of polyunsaturated lipids. The rate of lipid peroxidation, as determined by malonaldehyde appearance, was negligible without added porphyrin but increased with proto concentration in a dose-dependent fashion up to ~50 μM proto. $NaDodSO_4$ gel electrophoresis revealed that the initial rate of protein cross-linking likewise increased over this range. In the experiment with 1 μM proto, however, membranes producing no measurable malonaldehyde out to ~2 h of irradiation showed considerable cross-linking. This is illustrated by the 1-h sample (Figure 4, gel b). It would appear from these results that protein cross-linking can occur independently of peroxidation rather than as a consequence of it. However, this conclusion is subject to certain qualifications. For example, the inability to detect thiobarbituric acid color in irradiated membranes may not necessarily indicate that peroxide levels are negligible, since decomposing fatty acid peroxides generally give very low yields of malonaldehyde (Dahle et al., 1962). Also, since malonaldehyde is generated from fatty acid peroxides containing at least three double bonds (Dahle et al., 1962; Pryor et al., 1976), other species (including peroxides of cholesterol) would not be monitored in the malonaldehyde assay.

Effect of Antioxidants. Further evidence as to whether products of lipid peroxidation play any role in protein cross-linking was sought by the following approach. Membrane suspensions containing proto in sufficient concentrations to produce high levels of lipid peroxides were irradiated in the presence of antioxidants such as butylated hydroxytoluene (BHT) and *N,N'*-diphenyl-*p*-phenylenediamine (DPPD), and the effects on lipid and protein photodamage were assessed. Agents such as these can inhibit the propagation of lipid peroxidation by scavenging free radicals (Pryor et al., 1976) and have been used in vitro to block overall peroxidative damage in natural membranes and liposomes (Stocks & Dormandy, 1971; Leibowitz & Johnson, 1971; Anderson & Krinsky, 1973). The results of a typical experiment utilizing BHT are shown in Figure 5A. As can be seen, this agent was very effective in inhibiting malonaldehyde formation during irradiation. After a 1-h light dose, for example, the yield of malonaldehyde relative to that in a control without antioxidant was ~50% with 5 μM BHT and essentially zero with 10 times this concentration. The possibility that these effects were caused by interference of residual antioxidant with the thiobarbituric acid reaction was ruled out by showing that color produced by a malonaldehyde standard is unaffected by BHT, even at the highest concentration shown in Figure 5A. Furthermore, even though BHT has been reported to be a good trap for singlet excited oxygen in photodynamic systems (Thomas & Foote, 1978), it is unlikely that the above results can be dismissed on this basis (or on the basis of quenching of photoexcited sensitizer) since other types of photodamage were not inhibited by BHT (see below).

The disposition of the membrane proteins in the above experiment is shown in Figure 5B. In the control lacking antioxidant, cross-linking induced by 10 μM proto and a 1-h light dose is so extensive that only traces of bands 1–7 are left. Significantly, 50 μM BHT, which completely abolished malonaldehyde formation up to the 1-h point (Figure 5A), appeared to have no protective effect on cross-linking. Even 10 times this concentration was without effect. These observations are limited to a very advanced stage of cross-linking. In a separate experiment, however, the early time course of this reaction (as monitored by spectrin loss) was followed and was found to be essentially unaltered by BHT (data not shown). These results support the above contention that overall lipid peroxidation in this system is not casually related to protein cross-linking. It is clear from Figure 5B that this conclusion applies to all polypeptides, including those in band 3, which traverse the bilayer and would otherwise be expected to be more susceptible to peroxidation-mediated reactions than peripheral proteins such as spectrin.

The membrane damage described above was accompanied by extensive photoinactivation of intrinsic and extrinsic enzymes such as Na^+,K^+-ATPase, acetylcholinesterase, and

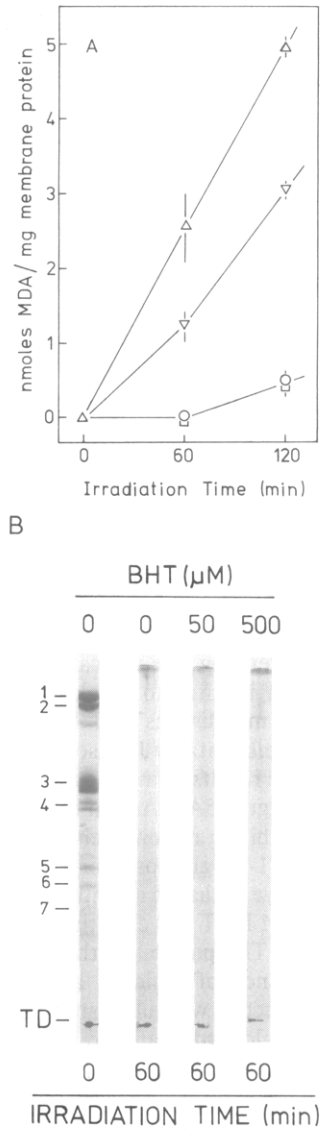

FIGURE 5: Lipid peroxidation and protein cross-linking in the presence of butylated hydroxytoluene (BHT). (A) Effect of BHT on malonaldehyde (MDA) formation. Ghosts (2.0 mg of protein/mL in 30P8 and 0.05% Tween-80) were preincubated for 15 min with BHT at the following concentrations: 0 (△); 5 (▽); 50 (○); and 500 μM (□). (Tween-80 was used to enhance the solubility of BHT and promote incorporation into the membranes.) The four preparations were then irradiated simultaneously in the presence of 10 μM proto. Samples were removed at the indicated times for determination of MDA. Values shown are means ± deviation of duplicate determinations. (B) Electropherograms of membrane proteins from the above preparations.

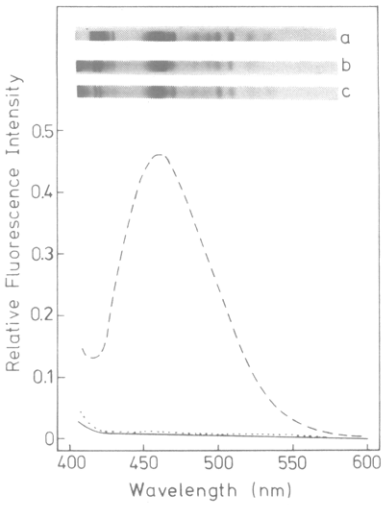

FIGURE 6: Fluorescence spectra of malonaldehyde (MDA)-treated and photooxidized ghosts. A membrane sample (2.0 mg of protein/mL in 6P8) was incubated in the dark with 9.1 mM MDA for 24 h at 25 °C. A second sample was irradiated for 1 h in the presence of 1 μM proto. Each preparation was solubilized in 3% $NaDodSO_4$ and 10 mM dithiothreitol (final protein concentration 1.0 mg/mL), and the fluorescence emission spectrum was recorded (excitation at 390 nm): (---) MDA-treated ghosts; (—) MDA alone or ghosts alone after 24 h of incubation; and (···) photooxidized ghosts. Inset shows $NaDodSO_4$ gels of the above preparations: (a) control lacking MDA; (b) MDA-treated ghosts; and (c) photooxidized ghosts.

glyceraldehyde-3-phosphate dehydrogenase (Table II). As a test of whether lipid peroxidation is implicated in these activity losses, an attempt was made to curtail these effects with antioxidants. It can be seen that BHT (0.1 mM) reduced malonaldehyde yield to <10%, but had no protecive effect whatsoever on Na^+,K^+-ATPase. Similarly, DPPD (0.1 mM) quenched peroxidation very efficiently, but offered no significant protection to acetylcholinesterase and glyceraldehyde-3-phosphate dehydrogenase. It is apparent, therefore, that photoinactivation of these enzymes, even those which are intimately associated with lipid (cholinesterase and the ATPase), cannot be ascribed to damaging attack by products of lipid peroxidation (e.g., peroxy radicals or malonaldehyde), nor can inactivation be a side effect of structural derangements in the peroxidized bilayer. Instead, active-site residues in these enzymes may be directly susceptible to photodynamic action. In addition, cross-linking reactions could play a role.

Although protein cross-linking is evidently not due to disulfide bridging, SH groups are nevertheless destroyed by photooxidation (Table I). Such losses were not affected by BHT under conditions in which this compound inhibited lipid peroxidation by >90% (Table II). As is true with cross-linking and enzyme inactivation, therefore, SH decay is probably not triggered by lipid reactions.

Cross-Linking with Malonaldehyde. Previous studies have demonstrated that malonaldehyde generated from its alkyl acetal can cross-link and inactivate soluble enzymes such as pancreatic ribonuclease (Chio & Tappel, 1969). The cross-links are presumably structures of the conjugated imine type, produced by coupling of malonaldehyde with two primary amino groups. Such structures have a fluorescence maximum at ~470 nm (excitation maximum at ~390 nm). A similar reaction was observed with malonaldehyde derived from autoxidized polyunsaturated fatty acids. It has been postulated, therefore, that this type of cross-linking also occurs in biomembranes under conditions of oxidative stress and that it is an important example of membrane damage in such systems (Tappel, 1975). Although such a mechanism could be invoked in the present case, the results obtained with antioxidants (Figure 5) argue against this possibility. As a further test of malonaldehyde involvement, photooxidized membranes were examined fluorometrically for the presence of characteristic conjugated imine cross-links. As seen in Figure 6, ghosts in which polypeptides had been extensively cross-linked by photodynamic action (gel c) showed negligible fluorescence in the 400–600-nm region. However, ghosts in which comparable levels of cross-linking had been produced by exogenous malonaldehyde (gel b) showed a strong visible fluorescence peak (emission maximum at 460 nm; excitation maximum at 390 nm). Cross-linked aminophospholipids as well as polypeptides could contribute to this peak. The absence of fluorescence in the irradiated membranes provides additional evidence against malonaldehyde-mediated cross-linking in such membranes.

Table II: Enzymatic Activity and Sulfhydryl Content of Membranes Irradiated in the Presence of Antioxidants[a]

antioxidant	time (min)	MDA (nmol/mg)	SH content (nmol/mg)	enzymatic activities: Na^+,K^+-ATPase [μmol/(mg h)]	enzymatic activities: AChE [μmol/(mg min)]	enzymatic activities: G3PD [μmol/(mg min)]
none[b]	0	0.05 ± 0.00[c]	84.3 ± 0.3[c] (100)	0.82 ± 0.06[c] (100)	2.92 ± 0.09[c] (100)	3.90 (100)
	30	0.42 ± 0.04	ND[d]	ND	1.08 ± 0.04 (37)	0.15 (4)
	60	1.27 ± 0.16	20.1 ± 2.3 (24)	0.14 ± 0.03 (17)	0.49 ± 0.00 (17)	0.14 (4)
BHT	0	0.00	89.6 ± 0.7 (100)	0.75 ± 0.02 (100)		
	30	0.02 (5)	ND	ND		
	60	0.12 (9)	20.6 ± 1.2 (23)	0.10 ± 0.04 (13)		
DPPD	0	0.05			2.94 ± 0.07 (100)	3.50 (100)
	30	0.02 (5)			1.24 ± 0.00 (42)	0.15 (4)
	60	0.10 (8)			0.63 ± 0.05 (21)	0.15 (4)

[a] Membranes [2.0 mg of protein/mL in 12 mM Tris-HCl (pH 8.0) and 0.05% Tween-80] were incubated with 100 μM butylated hydroxytoluene (BHT) or 100 μM *N,N'*-diphenyl-*p*-phenylenediamine (DPPD) and irradiated in the presence of 10 μM proto. Samples were removed for determination of malonaldehyde (MDA) and SH content and of activities of Na^+,K^+-ATPase, acetylcholinesterase (AChE), and glyceraldehyde-3-phosphate dehydrogenase (G3PD). Values shown are based on milligrams of membrane protein. Values in parentheses are as follows: (a) for MDA, percent formed relative to control after same light dose; (b) for SH, percent of zero-time content; (c) for enzymes, percent of zero-time activity. [b] Control. [c] Average ± deviation of values from duplicate experiments. [d] ND, not determined.

Discussion

Protoporphyrin-sensitized protein aggregation, as described here for isolated membranes, has also been observed in intact erythrocytes (Girotti, 1976a; Dubbelman et al., 1977). Essentially the same order of reactivity of different polypeptides is observed in both cases. In irradiated red cells, substantial photoaggregation of spectrin accompanies the cation leakage that ultimately results in osmotic swelling and hemolysis. At the same time, striking losses in cell deformability are seen (Dubbelman et al., 1977). These findings emphasize the importance of photoaggregation as a membrane-damaging event and have stimulated a growing interest in how this process takes place.

The fact that photoaggregated proteins resist dissociation under strenuous denaturing conditions, e.g., boiling in $NaDodSO_4$–urea mixtures (Figure 3), suggests that covalent cross-links are produced rather than unusually stable noncovalent interactions. This conclusion is reinforced by independent evidence obtained in earlier studies in which bilirubin was used as the photosensitizer (Girotti, 1978). Under identical reaction conditions proto is an exceedingly more potent sensitizer of cross-linking than bilirubin. However, the overall pattern of cross-linking obtained with the porphyrin (Figure 1A) is similar to that described with the bile pigment (Girotti, 1975, 1978). Polypeptide bands 1, 2, 4.1, and 6 on $NaDodSO_4$ gel electropherograms consistently disappear more rapidly during irradiation than do bands 3, 4.2, 5, and the sialoglycoproteins. Widely different photosensitizers, e.g., rose bengal and methylene blue, produce the same result (A. W. Girotti, unpublished observation). This suggests that the reactivity pattern observed is not governed by the sensitizer and how it interacts with the membrane, but by the organization of the proteins themselves. The fact that spectrin and band 4.1, for example, diminish rapidly may not be coincidental, since studies with cleavable cross-linking reagents (Wang & Richards, 1974) have shown that these proteins couple quite efficiently, evidently because of their association with one another on the inner membrane surface. A similar situation could apply to band 6, the subunit of glyceraldehyde-3-phosphate dehydrogenase, thereby explaining its relatively rapid disappearance. However, since this tetrameric enzyme has been reported to interact predominantly with band 3 rather than with spectrin (Yu & Steck, 1975), cross-linking between enzyme and band 3 or among subunits of the enzyme may be more important.

Up to this point, fading of a protein band during irradiation has been attributed entirely to losses due to cross-linking. It is important to consider whether alternative explanations are possible. One can imagine, for example, that the partial disappearance of relatively large peptides such as band 3 (cf. Figure 3, gel b) is due to physical trapping by extremely large photoaggregates of spectrin which remain at the gel origin. A similar problem was considered by Wang & Richards (1974) in chemical cross-linking experiments. In view of the following facts, however, trapping (if it occurs at all during electrophoretic analysis) could not have been very appreciable, at least in the experiment shown in Figure 3: (a) changes in the chromatographic profile following photooxidation mimic those seen electrophoretically; (b) diminution of a given protein peak (e.g., that near fractions 50–57, which represents band 3 primarily) must have been due to cross-linking and not to changes in assay sensitivity, since the integrated area under all protein peaks of the irradiated sample (fractions 20–75) differs from that of the control by <2%. Any significant trapping can also be ruled out on the grounds that peptides such as 4.1 and 4.2, which differ in size by only ~5% (Steck, 1972), disappear at far different rates.

Previous studies have shown that products of polyunsaturated fatty acid peroxidation, e.g., hydroperoxides themselves or byproducts such as malonaldehyde, can react with and inactivate a number of different enzymes, e.g., pancreatic ribonuclease, trypsin, and pepsin (Chio & Tappel, 1969; Gamage & Matsushita, 1973). One would anticipate, therefore, that the natural juxtaposition of certain proteins and unsaturated phospholipids in biological membranes would favor peroxidation-mediated damage to such proteins. In the present photodynamic system, however, this possibility has been ruled out, based on the following observations: (a) under certain conditions, extensive protein photodamage, e.g., cross-linking, precedes any significant formation of lipid peroxides, at least those which give rise to malonaldehyde (Figure 4); (b) isolated spectrin, though free of membrane lipids, is still susceptible to cross-linking (Figure 2); (c) although malonaldehyde generated from peroxidizing lipids can produce cross-linked proteins that have a characteristic visible fluorescence (Tappel, 1975), no such fluorescence is observed in photooxidized ghosts (Figure 6); (d) exogenous antioxidants such as butylated hydroxytoluene greatly inhibit lipid peroxidation but have no effect on cross-linking, enzyme inactivation, and loss of SH groups (Figure 5, Table II). Previous experiments showed that bilirubin-photosensitized aggregation of ghost proteins is also

unaffected by antioxidants (Girotti, 1978). Therefore, regardless of the type of photosensitizer used, it appears that protein photodamage in this system occurs independently of lipid peroxidation rather than as a side effect. A similar conclusion was reached by De Goeij et al. (1976), also studying protosensitized cross-linking in red cell membranes but employing a different approach from that described here. These workers reported, for example, that incubation of native ghosts with linoleic acid that had been extensively photoperoxidized by proto caused no detectable protein cross-linking.

No definitive evidence has been obtained yet about the types of amino acid residues that participate in cross-linking or the chemical nature of the bonds formed. However, certain possibilities can be eliminated, based on some of the present findings. For example, coupling of primary amino groups by malonaldehyde is ruled out. It is also unlikely that bifunctional lipid peroxy radicals (Roubal & Tappel, 1966) are acting as cross-linking moieties. This was confirmed in one of our experiments by determining lipid phosphate (Bartlett, 1959) in the column effluent (cf. Figure 3) of a membrane sample that had been photooxidized to a malonaldehyde level of ~ 4 nmol/mg of protein. Essentially all of the phosphate applied to the column emerged in the free phospholipid peak (fractions 62–77); negligible amounts (<1%) were found in the fractions containing protein aggregates and residual nonaggregated proteins (data not shown). It is apparent from these results that phospholipid peroxy radicals, once formed in this system, do not attack polypeptide chains to any appreciable extent.

The possibility that photoproducts of proto act as unique bridging groups appears remote for the following reasons: (a) similar cross-linking is observed with photosensitizers such as methylene blue, rose bengal, and bilirubin (Girotti, 1978); (b) cross-linking does not appear to be limited by proto concentration. For example, using a conservative estimate of 0.5 sensitizer consumed per spectrin subunit and neglecting reactions with other proteins, one would expect no more than about a 60% loss of bands 1 and 2 in the experiment shown in Figure 3 (compare gels a and b). The actual loss is obviously much greater than this, thereby arguing against cross-linking via sensitizer photoproducts.

Treatment of photooxidized ghosts with high concentrations of dithiothreitol (Figure 1) failed to reverse cross-linking to any appreciable extent, indicating that the predominant bonds formed are not -S-S- bonds. Moreover, blocking virtually all titratable SH groups in the membranes with *N*-ethylmaleimide prior to irradiation (Girotti, 1978) had no inhibitory effect on the reaction (data not shown). This rules out the possible involvement of SH groups in cross-linking other than -S-S- bond formation.

Clues about the nature of the cross-links produced in the present system may be gained from studies carried out with free amino acids. Tomita et al. (1969) showed that dye-sensitized photooxidation of histidine or *N*-benzoylhistidine gives a complex mixture of intermediates, many of which are converted to aspartic acid derivatives. These workers proposed a reaction mechanism in which some of the intermediates are conjugates generated by nucleophilic attack of residual substrate on oxidized imidazole groups. More recently, Straight & Spikes (1978) reported that photooxidation of the imidazole ring of histidine is accompanied by the disappearance of primary amino groups, as determined by fluorescamine assay. Nonphotooxidizable amino acids such as glycine, irradiated in the presence of *N*-acyl derivatives of histidine, also underwent a rapid loss of NH_2 groups, suggesting that some type of coupling occurs between these groups and initial oxidation products of the imidazole ring. Comparable effects were noticed with methionine, tyrosine, and tryptophan. A similar mechanism was recently proposed by Dubbelman et al. (1978) to account for the photoaggregation of red cell membrane proteins. In this proposal, proto-sensitized cross-linking is visualized as a secondary (light-independent) reaction between free NH_2 groups and photooxidation products of histidyl residues (and to a lesser extent tyrosyl and tryptophanyl residues). This scheme is based in part on the observation that blocking NH_2 groups with succinic anhdyride causes a dramatic reduction in cross-linking. Although one cannot completely rule out the above interpretation, it is severely clouded by the fact that extensive succinylation invariably causes polypeptides to unfold and dissociate because of electrostatic repulsion. Thus, a related anhydride, 2,3-dimethylmaleic anhydride, unfolds and elutes all extrinsic proteins from red cell ghosts (Steck & Yu, 1973), and this appears to be the basis of its protective action against bilirubin-sensitized cross-linking (Girotti, 1978). Recent experiments have revealed that extensive amidination (>90%) of NH_2 groups in isolated spectrin, a modification which causes minimal conformational changes, has no measurable effect on photosensitized cross-linking (A. W. Girotti, unpublished observation). These results contrast with those of Dubbelman et al. (1978) and suggest that free NH_2 groups (at least those existing at the onset of irradiation) may not be essential participants in the reaction. Additional studies aimed at a positive identification of such participants are currently underway in this laboratory.

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Isolation and Characterization of an Active Variable Domain from a Homogeneous Rabbit Antibody Light Chain†

P. H. Ehrlich, G. R. Matsueda, E. Haber,* and M. N. Margolies‡

ABSTRACT: The variable domain (V_L) of allotype b4 light chains of rabbit IgG was isolated from both nonimmune heterogeneous IgG and a homogeneous antibody directed against type III pneumococcal polysaccharide. Light chains were first isolated and then cleaved under mild acidic conditions between residues 109 and 110. Reduction with dithiothreitol in guanidine hydrochloride cleaved both intradomain disulfide bridges as well as the interdomain disulfide bridge joining the variable and constant domain. The sulfhydryl groups were protected after reduction by *p*-chloromercuribenzoate. V_L was isolated from this mixture of variable and constant domains by affinity chromatography, utilizing sheep antibodies directed against a peptide including residues 110–211 from nonimmune IgG light chain. The isolated V_L domain was identified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and automated Edman degradation. V_L from a homogeneous antibody was treated with dithiothreitol to remove *p*-chloromercuribenzoate, reoxidized, and recombined with homologous heavy chain. The binding of this recombinant to type III pneumococcal polysaccharide was identical with that of the light-chain–heavy-chain recombinant.

Immunoglobulin polypeptide chains are composed of linear repeating regions of homologous sequence, ~110–120 amino acid residues in length (Edelman, 1970). Sequence variability is located in the amino-terminal one-quarter of the heavy chain and the amino-terminal one-half of the light chain, suggesting that these regions constitute the antibody-combining site (Kabat, 1967). Their participation in antigen binding has been confirmed directly by X-ray crystallography (Segal et al., 1974; Poljak et al., 1973). X-ray studies have also supported the hypothesis that each homology region is an independent domain that has a tightly folded structure stabilized by a

† From the Departments of Medicine and Surgery, Harvard Medical School and the Massachusetts General Hospital, Boston, Massachusetts 02114. *Received March 22, 1979; revised manuscript received July 3, 1979.* Supported by National Institutes of Health Grants CA 24432 and HL 19259.
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