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PHOTODYNAMIC EFFECTS OF PROTOPORPHYRIN ON HUMAN ERYTHROCYTES

NATURE OF THE CROSS-LINKING OF MEMBRANE PROTEINS

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Summary

Protoporphyrin-sensitized photooxidation in human red blood cell membranes leads to severe deterioration of membrane structure and function. The membrane damage is caused by direct oxidation of amino acid residues, with subsequent cross-linking of membrane proteins.

The chemical nature of these cross-links was studied in model systems, isolated spectrin and red cell ghosts. Cysteine and methionine are not involved in the cross-linking reaction. Further it could be shown that dityrosine formation, the crucial mechanism in oxidative cross-linking of proteins by peroxidase-H_2O_2 treatment, plays no role in photodynamic cross-linking.

Experimental evidence indicated that a secondary reaction between free amino groups and a photooxidation product of histidine, tyrosine or tryptophan is involved in photodynamic cross-linking. This was deduced from the reaction observed between compounds containing a free amino group and photooxidation products of these amino acids, both in model systems, isolated spectrin and erythrocyte ghosts. In accordance, succinylation of free amino groups of membrane proteins or addition of compounds with free amino groups protected against cross-linking.

Quantitative data and consideration of the reaction mechanisms of photodynamic oxidation of amino acids make it highly probable that an oxidation product of histidine rather than of tyrosine or tryptophan is involved in the cross-linking reaction, via a nucleophilic addition by free amino groups.

Introduction

The photodynamic effects of protoporphyrin on human erythrocytes have been described in preceding papers [1—8]. These effects can be observed when

protoporphyrin is present inside the cells, as in erythropoietic protoporphyria, but also when protoporphyrin is added to the medium of normal erythrocytes [2,4]. It is highly probable that the photodynamic action involves the participation of singlet oxygen [9—12].

Irradiation of red blood cells in the presence of protoporphyrin leads among others to inhibition of several membrane-bound enzymes [13,14] and increased passive cation permeability of the membrane with subsequent osmotic hemolysis [2]. The membrane deterioration can be visualized by freeze-etch electron microscopy [4]. It has been shown that the membrane damage cannot be rationalized on the basis of peroxidation of unsaturated fatty acid residues of cholesterol in the membrane [5,6] but should be ascribed to photooxidation of amino acid residues of membrane proteins [6]. This photooxidation of amino acid residues leads to extensive cross-linking of membrane proteins. Especially spectrin is very sensitive to this protoporphyrin-induced cross-linking [4,6,8].

The amino acids, sensitive to protoporphyrin-induced photooxidation are cysteine, histidine, tyrosine, tryptophan and methionine [3]. Although several studies have been performed on the photooxidation of these individual amino acids, the chemical nature of the photodynamically formed cross-links of the membrane proteins is not clear. It has only been shown that this cross-linking is not caused by formation of disulfide bridges [6,8].

The present studies were designed to elucidate the chemical nature of the protoporphyrin-induced cross-linkages.

Methods

Heparinized human blood was centrifuged shortly after collection and washed three times in phosphate-buffered isotonic NaCl solution. Red cell ghosts were prepared according to the method of Weed et al. [15]. Spectrin was eluted from ghosts as described by Fairbanks et al. [16], precipitated at pH 5.1 [17] and further purified by gel chromatography on Sephadex G-200. Amino acid analysis was performed according to Dévényi [18] on a Beckman 120C amino acid analyzer equipped with a 6 mm sensitivity cell and a scale expander. Further analytical methods were: free sulfhydryl groups: following the method of Sedlak and Lindsay [19]; histidine: according to Sokolovsky and Vallee [20]; tyrosine: as described by Uehara et al. [21]; tryptophan: by the method of Spies and Chambers [22] and total protein: according to Lowry et al. [23]. Polyacrylamide gel electrophoresis of membrane proteins was performed as described by Fairbanks et al. [16] after dissolving the proteins in a solution containing 10 mM Tris, pH 8.0, 1 mM EDTA, 40 mM dithiothreitol and 1% sodium dodecyl sulphate.

Formation of dityrosine cross-links in proteins by treatment with peroxidase and hydrogen peroxide and quantification of dityrosine were done as described by Aeschbach et al. [24].

Illumination of solutions and cell suspensions in the presence of protoporphyrin was performed as described previously [4]. Reactions between amino acids, sensitive to photooxidation, and other amino acids were studied by illuminating mixtures of two amino acids in the presence of protoporphyrin. In these experiments one of the amino acids in the mixture was labeled with

^{14}C. Analysis of the reaction products was done via thin layer chromatography on silica gel with the solvent systems: *n*-butanol/acetic acid/water (4 : 1 : 1, v/v/v) and phenol/water (3 : 1, w/v) and subsequent autoradiography of the dried chromatogram. Radioactivity was assayed in a liquid scintillation counter. Photodynamic coupling of compounds to ghosts was measured by illumination of ghosts in the presence of these ^{14}C-labeled compounds and protoporphyrin. Subsequently the ghosts were washed five times with buffer and analyzed for radioactivity.

Succinylation of membrane protein amino groups was done by the procedure described by Habeeb et al. [25]. Oxygen consumption during photooxidation was measured with an YSI oxygen monitor equipped with a Clark-type electrode and a time-base recorder.

Results

Cross-linking of membrane proteins during illumination in the presence of protoporphyrin is shown in Fig. 1. With increasing illumination time a progressive accumulation of high molecular weight protein complexes on top of the gel can be observed, with a concomitant disappearance of the normal proteins from the gels. As discussed in detail elsewhere, spectrin bands 4.1 and 6 are more susceptible to cross-linking than the other membrane proteins, with the formation of covalent cross-linked protein complexes with apparent molecular weights above 10^6 [14]. These cross-links are not acid-labile; incubation of the ghosts at pH 1.9 for 20 min subsequent to illumination did not abolish the cross-linking.

A series of experiments was conducted to investigate the possible role of dityrosine formation in this cross-linking reaction. Protein cross-linking, caused by intermolecular dityrosine formation via enzymic oxidation has been described by several authors [24,26,27]. Enzymic oxidation of tyrosine, insulin and ribonuclease with peroxidase and H_2O_2 led to dityrosine formation. Dityrosine was detected by its characteristic fluorescence spectrum [24]. These experiments thus confirmed the results described in recent literature. Enzymatic oxidation of purified spectrin on the other hand, did not cause dityrosine formation. Moreover, protoporphyrin-induced photodynamic oxidation did not cause any detectable dityrosine formation with tyrosine, spectrin, insulin and ribonuclease. These results contradict a possible involvement of dityrosine bridges in photodynamic cross-linking.

The possible interaction of reaction products of photooxidizable amino acids with other amino acids was studied in model experiments. During illumination of photooxidizable amino acids in the presence of protoporphyrin a gradual decrease of the specific chemical reactions for these amino acids took place. With thin layer chromatography and autoradiography a proportional decrease of the amino acid with the concomitant formation of one or more oxidation products was observed. In these experiments illumination was continued until the photooxidizable amino acid had completely disappeared. Fig. 2 shows the results of photooxidation of [^{14}C]methionine. Identical autoradiograms were obtained with [^{14}C]methionine alone and with [^{14}C]-methionine mixed with a non-photooxidizable amino acid. Samples of [^{14}C]-

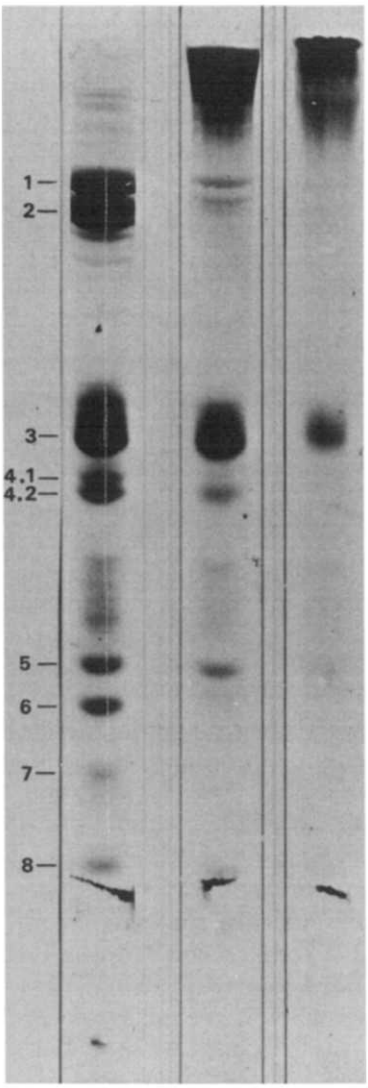


Fig. 1. Protoporphyrin induced cross-linking of erythrocyte membrane proteins as revealed by SDS polyacrylamide gel electrophoresis. Illumination times: 0—5—15 min. Protoporphyrin concentration: 0.05 mM; pH 7.5.

glycine, illuminated in the presence of protoporphyrin gave only one spot on autoradiograms with an R_f value corresponding to free glycine. Addition of unlabeled methionine to the reaction mixture had no effect (Fig. 3c). Similar results were obtained with the other non-photooxidizable amino acids serine, lysine and leucine. These results thus contradict an interaction between non-photooxidizable amino acids and the photooxidation products of methionine.

In a similar series of experiments with cysteine, instead of methionine, the same results were obtained. Again there was no indication of interaction between non-photooxidizable amino acids and photooxidation products of cysteine.

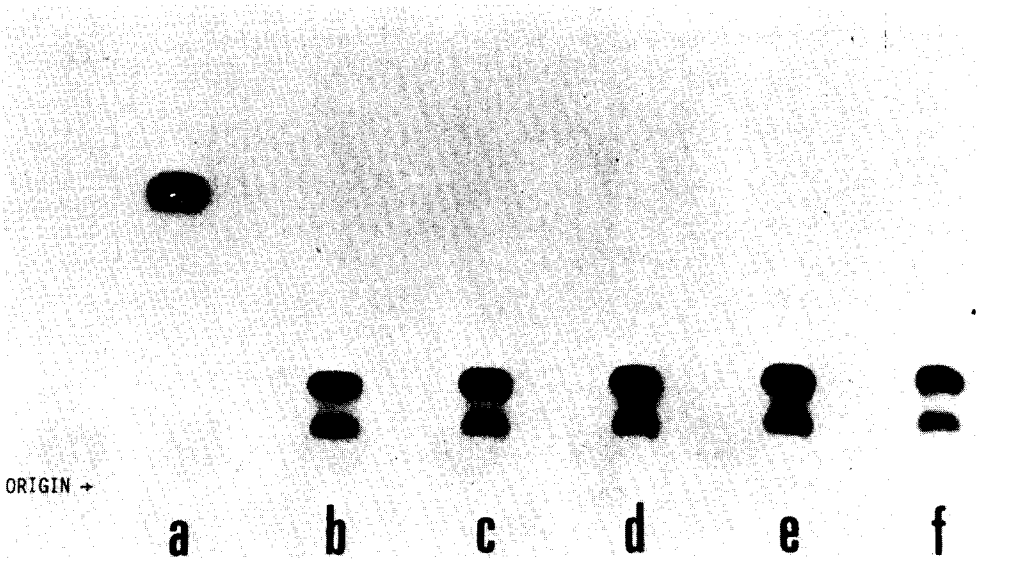


Fig. 2. Autoradiography of a silica gel thin layer chromatogram of photooxidation products of [^{14}C]-methionine, illuminated in the presence of protoporphyrin (0.3 mM) in phosphate buffer (10 mM, pH 9.0). The reaction mixture contained: (a) methionine (2 mM), not illuminated (reference); (b) methionine (2 mM); (c) methionine (2 mM) + glycine (2 mM); (d) methionine (2 mM) + glutamic acid (2 mM); (e) methionine (2 mM) + glutamine (2 mM); (f) methionine (2 mM) + cysteine (2 mM). Solvent system: butanol/acetic acid/water. With the solvent system phenol/water similar results were obtained.

In further experiments [^{14}C]glycine alone, or combined with one of the photooxidizable amino acids was illuminated in the presence of protoporphyrin. The results of a typical experiment are depicted in Fig. 3. With glycine alone, or combined with cysteine or methionine, only free glycine was present. Combined with histidine or tryptophan, and occasionally with tyrosine, additional spots could be detected. This indicates a reaction between glycine and photooxidation products of histidine and tryptophan and possibly tyrosine. It is clear from Fig. 3 that the reaction with histidine oxidation products is much more pronounced than with tryptophan and tyrosine oxidation products. Quantitative measurements of radioactivity revealed that under these experimental conditions 24.8, 4.9 and 2.9% of the glycine reacted with photooxidation products of respectively histidine, tryptophan and tyrosine. With [^{14}C]lysine, arginine, leucine, serine and ethylamine instead of [^{14}C]glycine, similar results were obtained. In all cases a weak reaction with tyrosine and tryptophan photooxidation products and a much stronger reaction with histidine-derived photooxidation products was observed (Table I). The fact that ethylamine was also reactive indicates that the NH_2-group is essential for the reaction. Considering these results, the possible reaction between hydroxylamine and the photooxidation products was of interest. It appeared that this agent reacts readily with photooxidation products of histidine, tryptophan and tyrosine.

A similar reaction appeared to occur during photooxidation between membrane proteins and [^{14}C]glycine, lysine and ethylamine, when ghosts were illuminated in the presence of protoporphyrin and varying concentrations of these agents. A Scatchard plot of the results showed that the total number of

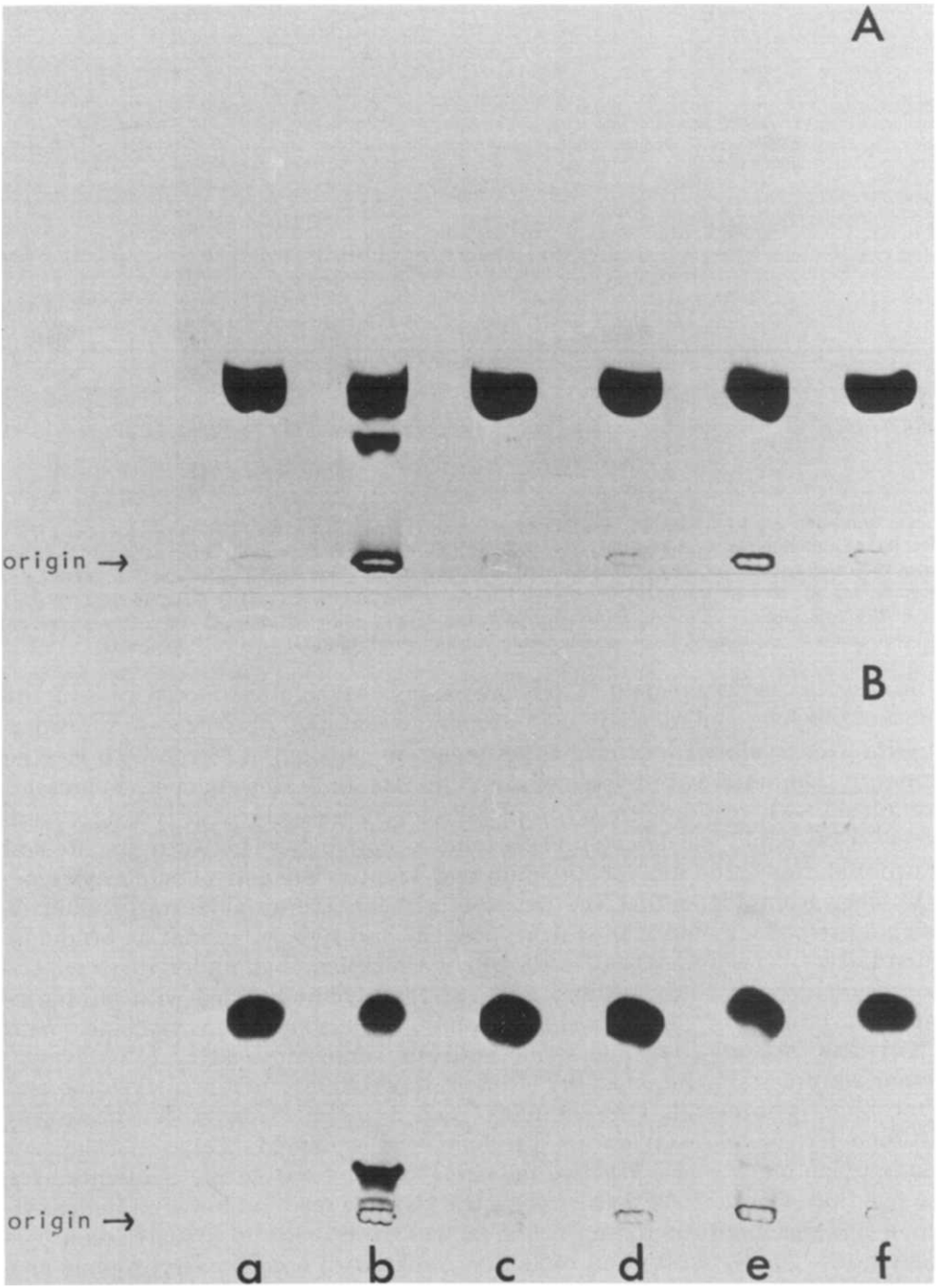


Fig. 3. Autoradiography of a silica gel thin layer chromatogram of [^{14}C]glycine, illuminated in the presence of a ———, b histidine, c methionine, d cysteine, e tryptophan, f tyrosine. Final concentrations of amino acids: 2 mM, of protoporphyrin: 0.3 mM and of phosphate buffer: 10 mM, pH 9.0. A: solvent system: butanol/acetic acid/water. B: solvent system: phenol/water.

TABLE I

REACTION OF NON-PHOTOOXIDIZABLE AMINO ACIDS AND ETHYLAMINE WITH PHOTOOXIDATION PRODUCTS OF HISTIDINE, TRYPTOPHAN, TYROSINE, METHIONINE AND CYSTEINE

Each of the above-listed compounds was present in a final concentration of 2 mM. Illumination was continued for 2 h in the presence of 0.3 mM protoporphyrin in 10 mM phosphate buffer, pH 9.0. Each value (percentage of the non-photooxidizable compound that has reacted) is the mean of 2 determinations.

	Histidine	Tryptophan	Tyrosine	Methionine	Cysteine
Glycine	24.8	4.9	2.9	0	0
Lysine	25.3	11.8	5.1	<1	<1
Serine	18,3	1.4	<1	0	0
Leucine	28.1	5.1	5.7	0	0
Ethylamine	31.7	8.9	4.0	0.6	0.5

reactive groups created by the photodynamic process was identical with all three amino compounds.

If the photodynamic cross-linking of membrane proteins depends on a similar reaction as observed in the model systems, photooxidation products of histidine, tryptophan and/or tyrosine residues in one polypeptide should be expected to react with free NH_2-groups in other polypeptides. This hypothesis was tested with several experimental approaches.

The amino acid composition of purified spectrin before and after illumination in the presence of protoporphyrin is given in Table II. The decrease of photooxidizable amino acid residues is shown in Fig. 4. Besides cysteine, histidine and tryptophan appeared to be photooxidized at an appreciable rate, whereas tyrosine did not decrease.

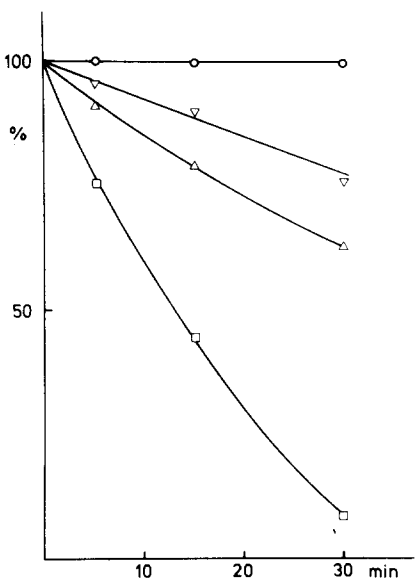
Based on the model experiments the inhibition of cross-linking by agents,

TABLE II

AMINO ACID COMPOSITION OF ISOLATED SPECTRIN BEFORE AND AFTER 30 MIN ILLUMINATION

For conditions see Fig. 4.

Amino acid	mol%	
	Before illumination	After illumination
Asp	10.8 ± 0.8	11.2 ± 0.3
Thr	4.4 ± 0.6	4.6 ± 0.4
Ser	7.2 ± 2.7	6.7 ± 0.7
Glu	17.2 ± 1.7	17.8 ± 0.5
Pro	2.8 ± 0.5	2.6 ± 0.2
Gly	7.5 ± 1.3	7.1 ± 0.6
Ala	8.9 ± 0.6	9.2 ± 0.2
Val	5.0 ± 0.4	5.0 ± 0.5
Met	2.1 ± 0.3	2.2 ± 0.4
Ile	4.2 ± 0.2	4.2 ± 0.3
Leu	10.0 ± 0.9	10.3 ± 0.5
Tyr	1.7 ± 0.2	1.6 ± 0.2
Phe	2.4 ± 0.3	2.6 ± 0.2
Lys	7.3 ± 0.8	7.5 ± 0.2
His	3.3 ± 0.5	2.1 ± 0.3
Arg	4.6 ± 0.5	4.5 ± 0.4
Trp	2.1	1.6



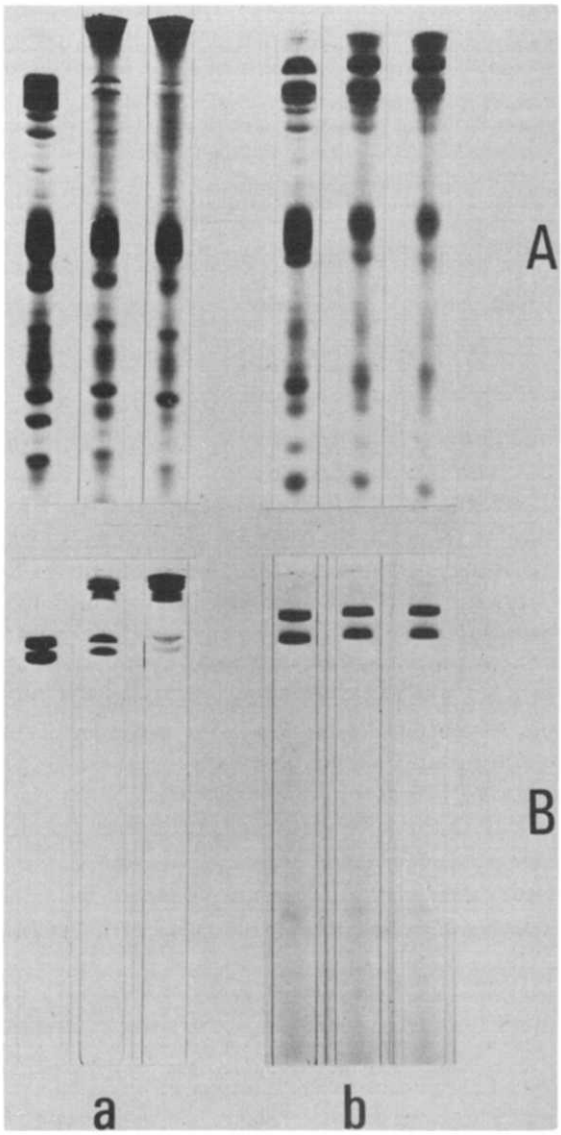


Fig. 4. Photooxidation of histidine (△), tryptophan (▽), tyrosine (○) and SH-groups (□) in isolated spectrin. Reaction mixture: 0.7 mg/ml spectrin, 0.05 mM protoporphyrin, and 10 mM phosphate buffer, pH 7.6. Each point is the average of at least three determinations.

Fig. 5. Protection against photodynamic cross-linking of erythrocyte membrane proteins (A) and isolated spectrin (B) after treatment with succinic anhydride. Ghost suspensions (2 mg protein/ml) and spectrin solutions (1 mg/ml) were illuminated for 0, 5 and 10 min in 10 mM phosphate buffer pH 8.0 in the presence of 0.05 mM protoporphyrin. a, control; b, treated with succinic anhydride (2 mg/mg protein; pH 8.0).

reacting with photooxidation products was studied. Both with ghosts and with isolated spectrin glycine, lysine and ethylamine yielded a slight degree of inhibition, whereas hydroxylamine gave a much stronger protection.

Finally, succinylation of membrane protein amino groups prior to photo-

oxidation yielded a very strong protection against photodynamic cross-linking in ghosts and in isolated spectrin (Fig. 5).

In control experiments it appeared that neither the addition of non-photooxidizable amino acids or amines nor succinylation of amino groups inhibited photooxidation of susceptible amino acid residues per se, as judged from measurements of oxygen consumption.

Discussion

The formation of covalent cross-links between membrane proteins in ghosts subsequent to protoporphyrin-sensitized photooxidation has been demonstrated in preceding studies [4,6,7,14]. The chemical nature of the cross-link was still obscure, but cannot be ascribed to the formation of disulfide bridges [8]. As shown in this paper dityrosine formation, as observed during oxidative cross-linking of several proteins by peroxidase-H_2O_2 treatment [24,27], could also be excluded as the molecular background of photosensitized cross-linking of ghost proteins.

The described experimental results strongly suggest that cross-linking is effectuated by a reaction between a photooxidation product of histidine residues and free amino groups in the involved polypeptide chains. This is based on the following observations.

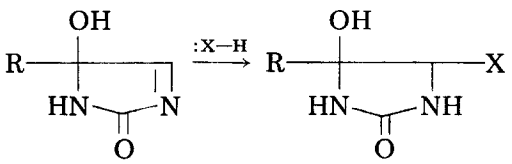
In the model experiments with mixtures of amino acids no reaction was observed between non-photooxidizable amino acids or amines on the one hand, and cysteine or methionine on the other. Reactions did occur with tyrosine, tryptophan and much more pronounced histidine (Table I). This suggests a reaction between a photooxidation product of one of these amino acids and free amino groups as the molecular mechanism for cross-linking, with histidine as the most probable source of the involved photooxidation product. Direct measurements of photooxidation of amino acid residues in spectrin (Table II, Fig. 4) support this supposition. From the results given in Table II and the apparent molecular weight of spectrin (about 230 000) it can be calculated that a spectrin polypeptide chain contains about 34 tyrosine, 66 histidine and 42 tryptophan residues. According to the results shown in Fig. 4 the number of photooxidized amino acid residues after an illumination period of 30 min (when cross-linking is virtually complete) is 0 for tyrosine, 26 for histidine and 10 for tryptophan. Although these results make it very unlikely that tyrosine would be involved in the cross-linking reaction, they do not fully exclude this possibility. Theoretically the reaction between only one photooxidizable residue on each spectrin polypeptide with one free NH_2-group on another chain would be sufficient for complete cross-linking. If tyrosine residues would be involved, this would require a minimal decrease of about 3%, which is within the experimental error.

The crucial role of free NH_2-groups in the cross-linking reaction was confirmed by the strong inhibition of cross-linking by succinylation of NH_2-groups prior to photooxidation (Fig. 5).

Based on the proposed mechanism it could be expected that photooxidation of ghost membrane proteins in the presence of amines or amino acids would result in covalent binding of these agents and, via competition, inhibition of

protein cross-linking. The experimental results were in accordance with this anticipation. Analysis of the results of binding experiments showed that the total number of reactive groups amounts to about 64 nmol/mg membrane protein. From the fact that spectrin comprises about 27% of the membrane protein [16,28] and the amino acid composition given in Table II it can be calculated that the spectrin included in 1 mg membrane protein contains about 77 nmol histidine, 49 nmol tryptophan and 40 nmol tyrosine. Although the amino acid composition of not all membrane proteins is known it can be expected that the total number of histidine, tryptophan and tyrosine residues per mg of membrane protein will be more or less proportional, thus roughly 3—4 times higher. On the other hand it should be expected that, due to sterical factors, many photooxidizable groups will not be reactive. Therefore, the value of 64 nmol/mg membrane protein may well be a reasonable reflection of the actual number of reactive groups.

Several studies on photosensitized oxidation of tyrosine, histidine and tryptophan appeared in recent literature [29—37]. Many photooxidation products of these amino acids have been identified. All three amino acids yield intermediates and products with aldehyde function. Reaction of free NH_2-groups with these aldehydes might yield Schiff's bases. However, it seems unlikely that this type of reaction would be responsible for the observed cross-links in the membrane proteins, considering their acid stability. The formation of a stable covalent bond by a reaction between free NH_2-groups and a particular intermediate of histidine photooxidation is more likely. This intermediate is very sensitive to nucleophilic addition at the C4 position [30]:



During photooxidation of histidine nucleophilic attack by the NH_2-groups as well as by imidazole residues at the C4 position readily occurs, leading to different final photooxidation products [29]. A similar reaction with NH_2-groups of other amino acid residues or amines seems very probable and could easily explain the experimental results described above. The very pronounced protection against cross-linking by the strong nucleophile hydroxylamine is in accordance with this interpretation.

Further experiments to clarify the exact mechanism of photodynamic cross-linking of membrane proteins are in progress.

Acknowledgments

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