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PHOTODYNAMIC PROTEIN CROSS-LINKING

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Exposure of spectrin to visible light in the presence of a photosensitizer results in photo-oxidation of sensitive amino acid residues and covalent cross-linking of the polypeptides. In a previous paper the cross-linking was ascribed to a secondary reaction between photo-oxidized histidine residues and amino groups. The following observations, described in this paper, are in accordance with this supposition. (1) During illumination of spectrin in the presence of a photosensitizer a pronounced photo-oxidation of histidine residues takes place. (2) Simultaneously a decrease of free amino groups is observed. (3) Semicarbazide protects against cross-linking and is bound to a histidine photo-oxidation product in spectrin. (4) The pH profile of histidine photo-oxidation and subsequent reaction with amino groups is similar to the pH profile of spectrin cross-linking. Amidination of NH_2 groups in spectrin does not inhibit cross-linking, as visualized by gel electrophoresis. On the other hand aminidation of denatured myoglobin causes a 50% inhibition of cross-linking. These observations support the notion of NH_2-involvement in cross-linking but also demonstrate, that other photodynamic cross-link mechanisms exist.

Introduction

Illumination of human erythrocyte membranes or spectrin in the presence of protoporphyrin with visible light leads to photo-oxidation of sensitive amino acid residues (cysteine, histidine, tyrosine, methionine and tryptophan) and covalent cross-linking of the protein molecules to high-molecular weight aggregates [1–3]. As shown before, the cross-linking is not a primary photodynamic event, but rather a secondary reaction between photo-oxidation products of susceptible amino acid residues and other groups of the protein molecule [4].

In a previous paper arguments were presented for a cross-link mechanism based on a reaction between photo-oxidized histidine and amino groups in the protein. This proposal was founded, among others, on model experiments in which photo-oxidation of histidine, tyrosine or tryptophan in the presence of ^{14}C-labeled, non-photooxidizable amines, yielded new ^{14}C-labeled products, presumably generated by a reaction between the photo-oxidized amino acid and the labeled amine [5]. Similar coupling reactions were observed between other nucleophilic agents, like semicarbazide, and the photo-oxidized amino acids. As histidine showed a much higher reactivity than tyrosine and tryptophan in these model experiments, it was tentatively concluded that mainly photo-oxidation products of histidine were involved in photodynamic protein cross-linking. A further argument was the protection against cross-linking by the same agents, reacting with photo-oxidized histidine in the model experiments. Utilizing the ^{14}C-labeled agents it appeared that a concomitant coupling of these small, protecting molecules to the protein occurred. Therefore the protective effect was interpreted as a competition between the protecting molecules and amino groups in the protein molecules, for reaction with photo-oxidized histidine [5,6].

Based on a quite different experimental approach Pooler and Valenzeno [7] also proposed a reaction between photo-oxidized histidine and amino groups as the cause of photochemical sodium channel block in lobster axons.

If the proposed model of photodynamic cross-linking is valid, the system would be expected to display the following properties. (1) During illumination of spectrin in the presence of a sensitizer a decrease of histidine residues should occur. (2) Simultaneously the number of free amino groups should decrease. (3) Protection against cross-linking by amines and other nucleophilic agents should be reflected by binding of these molecules to photo-oxidized histidine residues. (4) The pH-dependence of histidine photo-oxidation and subsequent reaction with NH_2-groups should be similar to the pH-dependence of spectrin cross-linking. (5) Amidination of amino groups should inhibit cross-linking.

These predictions were tested experimentally as described in the present communication.

Methods and Materials

Heparinized human blood was centrifuged and the red blood cells were washed three times with phosphate-buffered isotonic NaCl. Red cell membranes were prepared by the gradual osmotic lysis method of Weed et al. [8]. Spectrin was extracted from the ghosts according to Bennett and Branton [9], purified by precipitation at pH 5.1 [10] and dissolved in 10 mM phosphate buffer.

Illumination in the presence of a sensitizer was carried out as described before [1]. Unless otherwise stated the protein concentration was 1 mg/ml with either 0.06 mM protoporphyrin or methylene blue. Illumination of photosensitive amino acids in model experiments was done with solutions containing 2 mM aminoacid and 0.1 mM sensitizer.

Photodynamic coupling of semicarbazide to spectrin was done by illumination of the protein in 10 mM phosphate buffer, pH 8.5, in the presence of 0.06 mM protoporphyrin and varying concentrations of ^{14}C-labeled semicarbazide. After illumination samples were dialyzed against 10 mM phosphate buffer, pH 7.0, to remove unbound semicarbazide. Subsequently the sample was either analyzed for radioactivity with a liquid scintillation counter or hydrolyzed.

Enzymatic hydrolysis of spectrin was performed by incubation at 37°C for 24 h with pronase (0.5 mg per mg protein), subsequently for 24 h with aminopeptidase M (0.5 mg per mg protein) and finally for 24 h with carboxypeptidase A (0.5 mg per mg protein). The hydrolysate was purified by gel filtration on a Sephadex G-25 (Superfine) column (1.25 × 26 cm). Elution was done at room temperature with H_2O/NH_4OH, pH 8.1, at a rate of 50 ml/h, collecting 2-ml fractions. The fractions containing free amino acids were pooled and analyzed by thin-layer chromatography. Thin-layer chromatography was performed on silica gel, with the solvent systems *n*-propanol/25% NH_4OH (7 : 3, v/v) and phenol/H_2O (3 : 1, w/v) with subsequent autoradiography of the dried chromatogram.

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis of spectrin was performed according to Fairbanks et al. [11]. Densitometric scans of the Coomassie Blue stained protein bands were recorded with a Zeiss PMQ II spectrophotometer with scanning device at 620 nm. The disappearance of Coomassie Blue from the spectrin position in the electropherograms was taken as a direct measure of spectrin cross-linking. This is justified as photooxidation of the protein does not reduce Coomassie Blue staining. This was shown e.g. in model experiments on succinylated spectrin, which is photooxidized with the same velocity as untreated spectrin, but without cross-linking [5].

Protein concentrations were measured according to the method of Lowry et al. [12] with bovine serum albumin as a protein standard.

Histidine was determined according to Sokolovsky and Vallee [13], tryptophan as described by Spies and Chambers [14] and tyrosine according to Uehara et al. [15]. Free NH_2-groups were assayed with the fluorescamine method described by Udenfriend et al. [16]. Amidination of NH_2 groups was carried out as described by Girotti [17].

Materials. All reagents were analytical grade and used without further purification. ^{14}C-labeled semicarbazide was obtained from ICN Pharmaceuticals (Irvine). Pronase, aminopeptidase M and carboxypeptidase A were obtained from Boehringer (Mannheim).

Results

Photodynamic effects on free amino acids

During illumination of histidine in the presence of protoporphyrin and ^{14}C-labeled glycine, a new ^{14}C-labeled product was formed (Fig. 1, b). This product

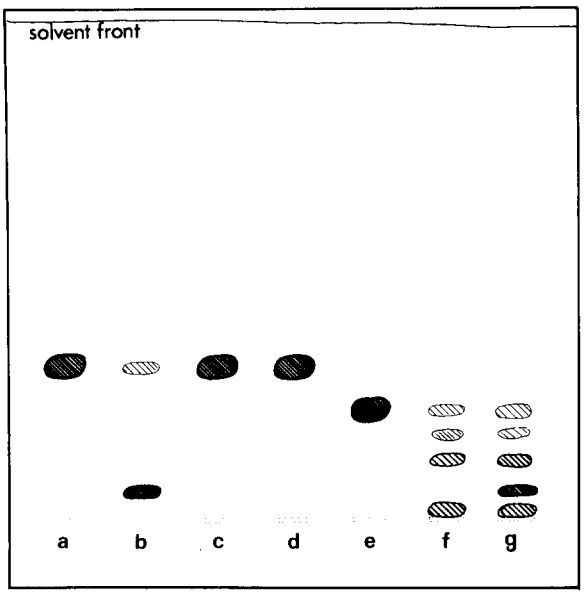

Fig. 1. Autoradiography of silicagel thin-layer chromatograms of ^{14}C-labeled glycine (a, b, c, d) and ^{14}C-labeled histidine (e, f, g). Solvent system: phenol/H_2O (3 : 1, w/v). (a) untreated glycine; (b) after illumination in the presence of protoporphyrin with histidine; (c) as (b) without histidine; (d) as (b) after treatment of the illuminated mixture with 1 M HCl at 100°C for 1 h; (e) untreated histidine; (f) after illumination in the presence of protoporphyrin; (g) as (f) with glycine.

was not formed when histidine was omitted from the reaction mixture (Fig. 1, c). In a previous paper these results were tentatively ascribed to a coupling reaction between histidine photo-oxidation products and the NH_2-groups of the labeled compounds [5]. To reinforce this supposition, additional experiments were performed.

Treatment of the illuminated reaction mixture with 1 M HCl at 100°C during 1 h resulted in the complete disappearance of the coupling products. All radioactivity was recovered again at the position of the original ^{14}C-labeled compound (Fig. 1, d). This is in accordance with the notion of an (apparently acid-labile) coupling product.

After illumination of ^{14}C-labeled histidine in the presence of protoporphyrin, several oxidation products were recovered after thin-layer chromatography (Fig. 1, f). When non-labeled glycine was added to the reaction mixture, an additional spot was observed (Fig. 1, g) with the same R_F value as the spot in Fig. 1, b. These results confirm again the supposition that coupling occurs between a histidine photo-oxidation product and the non-photo-oxidizable glycine.

With semicarbazide (instead of glycine) the results were slightly different in the sense that three coupling products were found with photo-oxidized histidine (see Fig. 5, A). A possible explanation is the fact that photo-oxidation of histidine yields several different intermediates and products [18,19]. It is conceivable that more than one of the intermediates is reactive towards semicarbazide.

When histidine was replaced by tryptophan or tyrosine, similar coupling products were formed, but in lower final yield. With methionine and cysteine as photo-oxidizable amino acids no coupling products with glycine or semi-carbazide were recovered.

During illumination of histidine, tyrosine or tryptophan in the presence of a sensitizer, the amino acids are gradually destroyed, as measured by the decreased reactivity with specific reagents. Simultaneously a decrease of free amino groups is observed (Fig. 2) as described before by Straight and Spikes [20]. As

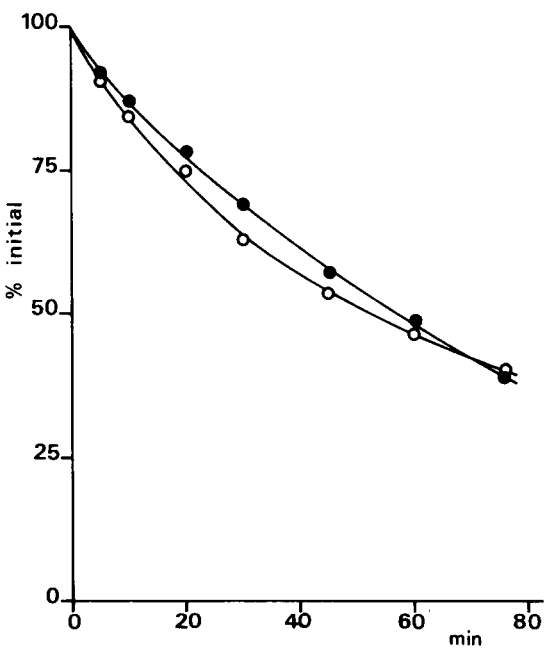

Fig. 2. Photo-oxidation of histidine at pH 8.6 in the presence of protoporphyrin. Curves show decrease of histidine (●——●) and NH_2 (○——○).

amino groups themselves are not photo-oxidizable, it is highly probable that the decrease of amino groups reflects a coupling reaction between photo-oxidized side chains and NH_2-groups of the starting material. It should be realized that with free amino acids this reaction does not necessarily lead to dimerization. With histidine, for instance, the reaction may also occur between an oxidized imidazole and the NH_2 group of the same molecule. Further conversions of these reaction products lead to different, stable final products, as shown in detail by Tomita et al. [18,19].

The influence of pH on photo-oxidation of histidine, tyrosine and tryptophan and on the concomitant decrease of amino groups is shown in Fig. 3. Both titration of the sensitizer and titration of the photo-oxidizable amino acid side chains will contribute to the pH dependency of photo-oxidation. Therefore these studies were performed both with protoporphyrin and with methylene blue as sensitizer, to facilitate analysis of the results, which show rather characteristic profiles for each of the amino acids. For instance a pH shift from 10 to 11 yields a strong increase of tyrosine photo-oxidation with both sensitizers. This can not be attributed to titration of the

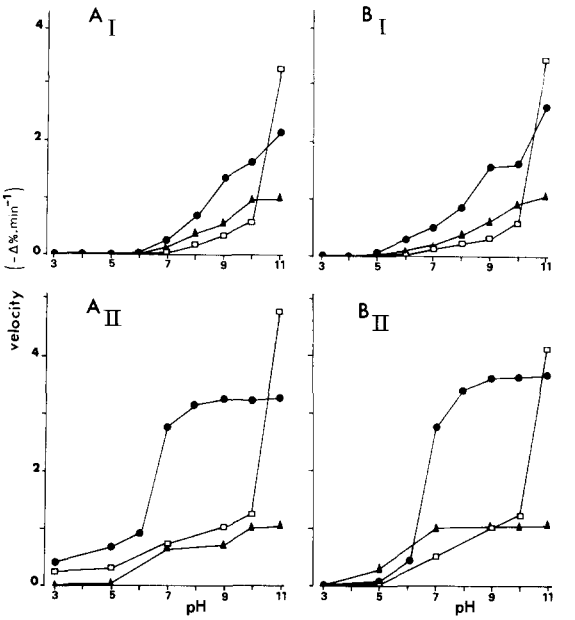

Fig. 3. A. Photo-oxidation of histidine (●———●), tryptophan (▲———▲) and tyrosine (□———□) at different pH with protoporphyrin (I) and methylene blue (II) as sensitizers. B. Decrease of NH_2-groups in a solution of histidine (●———●), tryptophan (▲———▲) and tyrosine (□———□) at different pH with protoporphyrin (I) and methylene blue (II) as sensitizers.

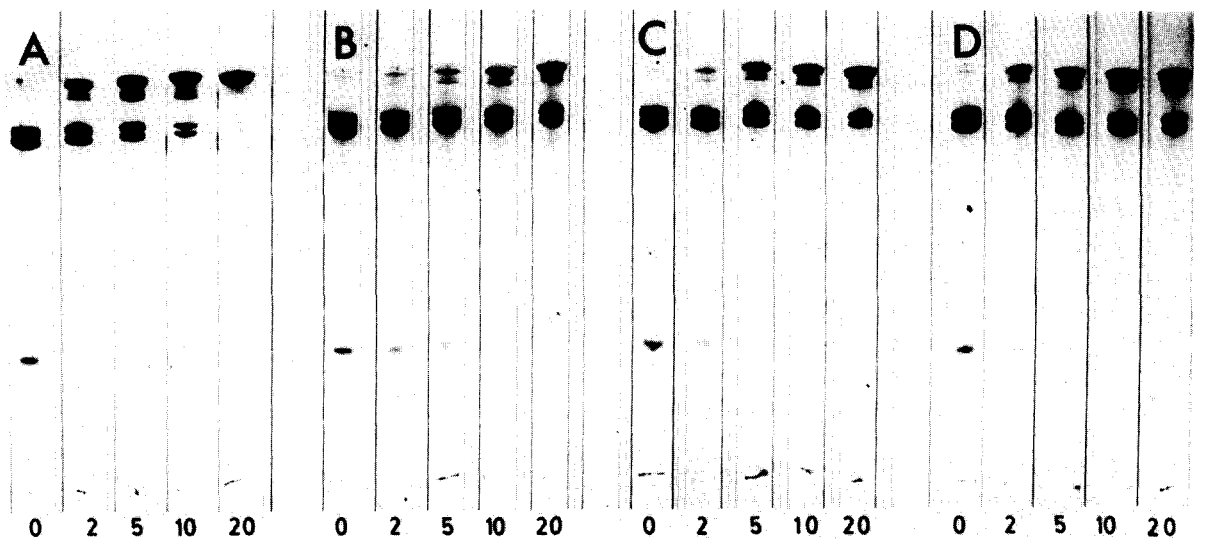

Fig. 4. SDS-gel electrophoresis of spectrin. Spectrin solutions were illuminated in 10 mM phosphate buffer, pH 8.6, in the presence of protoporphyrin for the indicated times (minutes). (A) control; (B) with 50 mM hydroxylamine; (C) with 50 mM semicarbazide; (D) with 50 mM ethylamine.

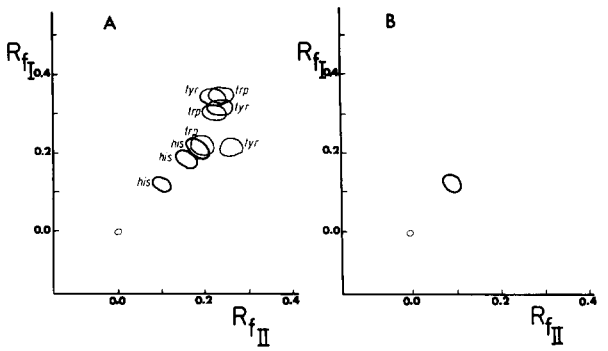

Fig. 5. Autoradiography of two-dimensional silica gel thin-layer chromatograms of ^{14}C-labeled semicarbazide. Solvent systems: (I) *n*-propanol/25% ammonia (7 : 3, v/v) and (II) phenol/water (3 : 1, w/v). A. Binding products of semicarbazide with histidine, tryptophan and tyrosine after illumination in the presence of protoporphyrin. B. Binding product of semicarbazide with spectrin after illumination in the presence of protoporphyrin and subsequent purification of the binding product as described in Methods.

sensitizer, as photo-oxidation of the other amino acids is hardly effected by this pH shift. Fig. 3 also shows that the pH profiles of photo-oxidation and NH_2-decrease are quite similar for each of the amino acids, suggesting a close correlation between these two processes.

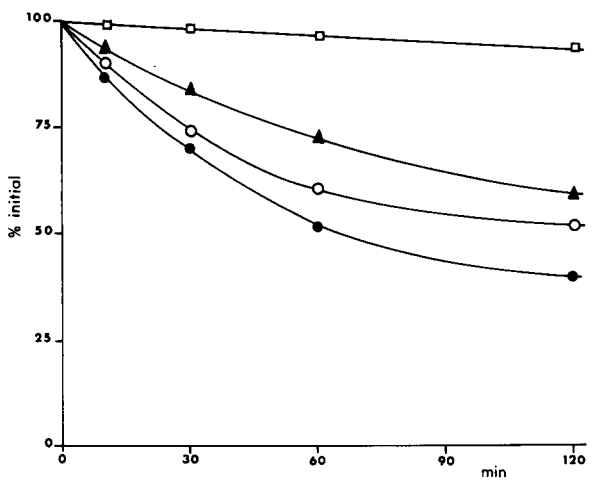

Fig. 6. Photo-oxidation of tyrosine (□———□), tryptophan (▲———▲) and histidine (●———●) residues in spectrin at pH 8.6 in the presence of protoporphyrin. Also shown is the loss of NH_2-groups (○———○) assayed with the fluorescamine method. Initial values according to Shotton et al. [22]: tyrosine, 183 nmol/mg; tryptophan, 152 nmol/mg; histidine, 248 nmol/mg; amino groups, 580 nmol/mg.

Photodynamic effects on spectrin

Photodynamic cross-linking of spectrin and protection against cross-linking by hydroxylamine, semicarbazide and ethylamine are shown in Fig. 4. The inhibition of cross-linking by these compounds is not caused by inhibition of photo-oxidation. Photo-oxidation of free amino acids and of susceptible residues in spectrin was uneffected by hydroxylamine, semicarbazide and ethylamine. In previous studies it has been shown that these small, protecting molecules are covalently bound to the protein molecule during illumination in the presence of a sensitizer. According to the method of calculation described previously [6] a maximal binding of 55 nmol semicarbazide/mg spectrin was found.

In an attempt to identify the semicarbazide binding groups in spectrin, the protein was illuminated in the presence of ^{14}C-labeled semicarbazide and protoporphyrin during 2 h. Excess, unreacted semicarbazide was removed by extensive dialysis. Subsequently the protein was hydrolyzed enzymatically, as described in the methods section. Two-dimensional thin-layer chromatography now revealed a radioactive spot with R_F values, corresponding to a semicarbazide-photo-oxidized histidine coupling product, as found in model experiments (Fig. 5). The correspond-

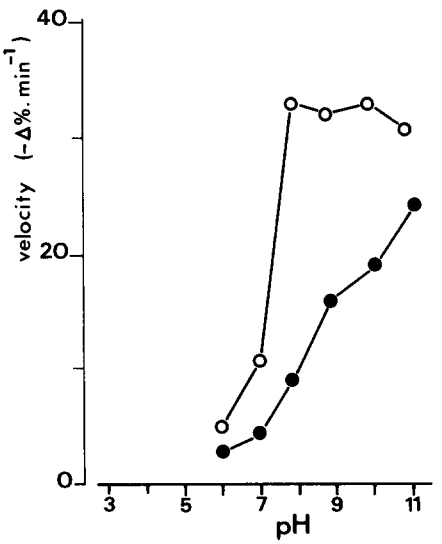

Fig. 7. Photodynamic cross-linking of spectrin at different pH in the presence of protoporphyrin (●———●) and methylene blue (○———○). The initial velocity of spectrin decrease is expressed as $-\Delta\% \cdot min^{-1}$ as calculated from densitometric scans of band 1 and 2 (spectrin monomers).

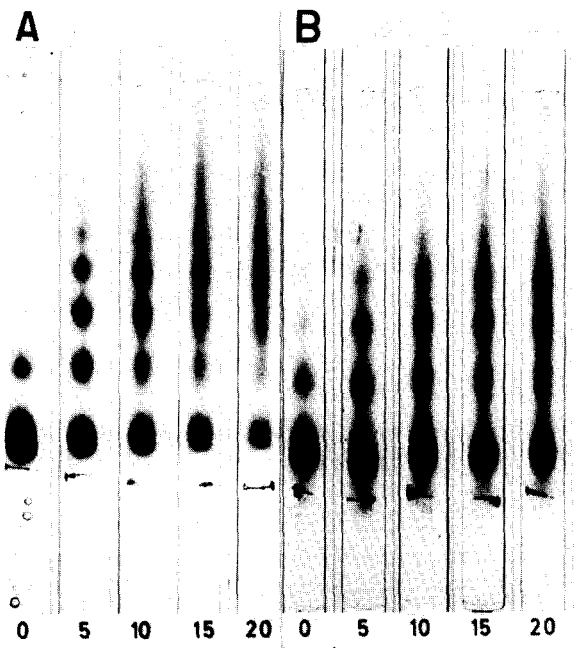

Fig. 8. A. SDS gel electrophoresis of myoglobin, illuminated in 8 M urea, pH 8.6, in the presence of methylene blue for the indicated times (min). B. As A, after amidination of the protein.

ing coupling products of semicarbazide with photo-oxidized tryptophan and tyrosine showed significantly different R_F values (Fig. 5). Fig. 6 shows the disappearance of histidine, tryptophan, tyrosine and NH_2 groups during protoporphyrin-sensitized photo-oxidation of spectrin. After 2 h of illumination about 60% of the histidine residues, 40% of the tryptophan residues and less than 6% of the tyrosine residues had been destroyed. Simultaneously a pronounced decrease of NH_2 groups was observed.

Finally the pH dependency of photodynamic cross-linking of spectrin was studied over the pH range 6–11, with both protoporphyrin and methylene blue as sensitizers. The results are shown in Fig. 7.

Effect of amidination on cross-linking

Amidination of about 90% of the NH_2-groups in spectrin did not inhibit cross-linking as visualized by SDS-polyacrylamide gel electrophoresis (not shown). Native myoglobin did not exhibit photodynamic interpeptide cross-linking. The protein was cross-linked readily, however, when illuminated in 8 M urea in the presence of a sensitizer. Under these experimental conditions amidination of 95–100% of the NH_2-groups caused about 50% inhibition of cross-linking (Fig. 8).

Discussion

In a previous paper Girotti has presented evidence that sulfhydryl groups are not involved in photodynamic cross-linking [17]. In the present studies the attention was focussed on other photo-oxidizable amino acid residues.

Model experiments, as depicted in Figs. 1–3, clearly show a reaction between amino groups and histidine, tryptophan and tyrosine photo-oxidation products, leading to covalent coupling. A similar reaction in proteins, causing covalent intra- or interpeptide cross-links, is highly probable and affirmed by the decrease of non-photo-oxidizable amino groups during illumination of spectrin in the presence of a sensitizer (Fig. 6). Under these experimental conditions spectrin has disappeared from its normal position in the electropheretogram in about 10 min, meaning that at that moment each spectrin monomer is involved in at least one intermolecular cross-link. Taking the data of Fig. 6, the decrease of histidine, tryptophan, tyrosine and amino groups at that point amounted to 13, 6, 0.4, and 10%, respectively. Based on the amino acid composition of spectrin according to Shotton et al. [22] this corresponds to 8 histidine, 2 tryptophan, less than 0.2 tyrosine and 13 amino groups per monomer. These figures should be considered with caution. The percentual decrease between 0 and 10 min is only small and the indicator reactions do not necessarily change linearly with the functional group concentrations. Despite these limitations the data virtually exclude a major role of tyrosine residues in these early cross-linking reactions and support an important role of histidine residues. The probable role of histidine in early cross-linking as reflected by SDS polyacrylamide gel electrophoresis is further supported by two additional observations. Firstly the pH profiles of amino acid destruction in model experiments (Fig. 3) and spectrin cross-linking (Fig. 7) reveal a much closer parallel between cross-linking and histidine photo-oxidation than between cross-linking and tryptophan or tyrosine photo-oxidation, both with protoporphyrin and with

methylene blue as sensitizer. Although it should be realized that photo-oxidation of sensitive residues in proteins may show different characteristics as compared to the free amino acids, the results suggest again the involvement of histidine in cross-linking.

Secondly, [^{14}C]semicarbazide, bound to spectrin during illumination in the presence of protoporphyrin, is recovered from the hydrolysate as a semicarbazide-histidine reaction product (Fig. 5). As the inhibition of cross-linking by semicarbazide can not be attributed to inhibition of photo-oxidation, the most likely interpretation is interference with the secondary cross-linking reaction by competition, as discussed previously [5,6]. Reaction of semicarbazide with photooxidized amino acid residues will prevent reaction of these residues with reactive groups in the protein molecules, thus inhibiting cross-linking. The results shown in Fig. 5 therefore strongly suggest an important role of histidine in early cross-linking. The maximal binding of semicarbazide amounted to 55 nmol/mg spectrin, after an irradiation time of 2 h. This demonstrates that a large proportion of the histtidine and tryptophan residues, photo-oxidized in this period (about 150 and 60 nmol, respectively), is not reactive towards semicarbazide, presumably due to steric factors.

Although the most spectacular effect of photodynamic cross-linking is the change in the electrophoretic pattern during the first 10 min, the measurements between 10 and 120 min in Fig. 6 are more important from a quantitative point of view. Not only photo-oxidation of histidine, tryptophan and, to a much less extent, of tyrosine, but also the disappearance of the non-photo-oxidizable amino groups proceeds. The only obvious interpretation of these observations is that in this period many additional inter- and/or intramolecular cross-links are formed between amino groups and photo-oxidized residues.

Although these results, together with the above mentioned observations of Straight and Spikes [20] and Tomita et al. [18,19] strongly support the notion photodynamic cross-links between amino groups and photo-oxidized amino acid residues, they do not exclude other mechanisms of cross-linking. In this context recent observations of Girotti [17] are important. It was shown that cross-linking of spectrin, as reflected by SDS-polyacrylamide gel electrophoresis, was not inhibited by amidination of 95% of the NH_2-groups [17]. As mentioned in the results section, we could confirm these observations. These experiments most probably indicate that other cross-link reactions can also occur, assuming that the rate of photo-oxidation rather than the velocity of the secondary cross-linking reactions is rate-limiting with respect to the changes visualized by SDS-polyacrylamide gel electrophoresis. This interpretation is strongly supported by the results shown in Fig. 8. Cross-linking of myoglobin in 8 M urea is about 50% inhibited by amidination, clearly showing NH_2-involvement in photodynamic cross-linking of this protein. On the other hand cross-linking is not completely abolished, indicating that other cross-link reactions occur.

Further experiments to elucidate these other cross-link mechanisms are presently under way in this laboratory.

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