Biochimica et Biophysica Acta, 602 (1980) 45—56

BBA 78961

PHOTOSENSITIZED CROSS-LINKING OF ERYTHROCYTE MEMBRANE PROTEINS

EVIDENCE AGAINST PARTICIPATION OF AMINO GROUPS IN THE REACTION

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(Received May 9th, 1980)

Key words: Spectrin; Cross-linking; Photosensitizer; Dithiothreitol; Ethylamine; (Erythrocyte membrane)

Summary

Exposure of human erythrocyte ghosts (pH 8, 10°C) to visible light in the presence of the photosensitizer, methylene blue, results in a relatively rapid loss of spectrin (bands 1 and 2 on sodium dodecyl sulfate gel electropherograms) and the appearance of high molecular weight cross-linked derivatives. Isolated spectrin also undergoes photosensitized cross-linking, indicating that the reaction is not lipid-dependent.

Extensive cross-linking was neither reversed by dithiothreitol nor prevented by prior blocking of SH groups with *N*-ethylmaleimide, suggesting that cysteine residues are not crucial bridging sites. The possible requirement for NH_2 groups, as suggested by previous model studies (Dubbelman, T.M.A.R., de Goeij, A.F.P.M. and van Steveninck, J. (1978) Biochim. Biophys. Acta 511, 141—151), was tested. Succinylation of spectrin protected against cross-linking, but this effect is attributed to the disruption of quaternary structure, as deduced from sedimentation measurements. However, virtually complete blocking of NH_2 groups by amidination perturbed overall structure relatively little, and had no effect on cross-linking. Moreover, exogenous amines such as ethylamine, added in large excess to spectrin prior to irradiation, did not interfere with cross-link formation. These results suggest that NH_2 groups are not involved in the reaction.

Abbreviation: SDS, sodium dodecyl sulfate.

Introduction

It is well known that photosensitized oxidation of biological membranes is deleterious to membrane structure and function [1,2]. In an earlier publication, I reported a striking and previously undescribed effect of photo-oxidation on biomembranes [3]. Continuous blue-light irradiation of human erythrocyte ghosts in the presence of O_2 and the bile pigment, bilirubin, resulted in an apparent cross-linking of membrane proteins, as visualized by SDS-polyacrylamide gel electrophoresis. Working independently, de Goeij et al. [4] observed a similar effect, using protoporphyrin IX as the photosensitizing agent. Follow-up studies by these workers [5] and myself [6] showed that cross-linking of membrane proteins also takes place in intact erythrocytes, and precedes to a large extent the swelling and hemolysis that these cells undergo under photodynamic stress. Photodamage in the form of cross-linking could result in a variety of adverse secondary effects, including hindered membrane fluidity, inactivation of intrinsic enzymes and reduced deformability. In the present report, the susceptibility of erythrocyte membrane proteins to photosensitized cross-linking by a synthetic dye, the thiazine methylene blue, is described. One of the primary objectives of the work has been to identify the types of amino acid residue that participate in the reaction, or at least to rule out certain possibilities.

Materials and Methods

Materials. Methylene blue trihydrate was obtained from Matheson, Coleman and Bell (Cincinnati, OH). Isethionyl acetimidate hydrochloride was a product of Pierce Chemical Co. (Rockford, IL). Ethyl acetimidate hydrochloride and glutaraldehyde (25%) were from Aldrich Chemical Co. (Milwaukee, WI). Sigma Chemical Co. (St. Louis, MO) provided the fluorescamine, *N*-ethylmaleimide, 5,5'-dithiobis(2-nitrobenzoic acid) and phenylmethanesulfonyl fluoride. Succinic anhydride and the reagents used for SDS-polyacrylamide gel electrophoresis were from Eastman Organic Chemicals (Rochester, NY).

Human blood in citrate/phosphate/dextrose medium was obtained from the Milwaukee Blood Center and used within 2 weeks. Erythrocyte ghosts were prepared as described previously [3]. Extraction of spectrin (polypeptide bands 1 and 2 on SDS-polyacrylamide gel electropherograms) from freshly prepared ghosts was carried out according to the method of Fairbanks et al. [7], with slight modifications. Stock membranes in 6 mM sodium phosphate (pH 8.0) were diluted with 19 vols. of a pH 8.5 solution containing 0.1 mM EDTA and 20 μM phenylmethanesulfonyl fluoride, and incubated for 15 min at 37°C. The supernatant solution recovered after centrifugation at 40 000 $\times g$ for 45 min was dialyzed at 4°C against a buffer containing 50 mM sodium phosphate (pH 7.7), 100 mM NaCl, 0.1 mM EDTA and 2 mM β-mercaptoethanol. Immediately before being used in chemical-modification reactions, the solution (containing spectrin and traces of band 5) was concentrated approx. 5-fold in a hollow-fiber apparatus (Bio Rad Laboratories) and dialyzed against 50 mM sodium phosphate (pH 8.0), 0.1 mM EDTA.

Protein concentrations were determined according to the method of Lowry

et al. [8], using bovine serum albumin as the standard.

Chemical modification reactions. Sulfhydryl groups in spectrin were blocked as follows. The protein (0.2 mg/ml) was incubated with 5 mM *N*-ethylmaleimide for 1 h at 25°C, followed by dialysis against 50 mM phosphate (pH 8.0). The SH content of native and modified spectrin was determined by a variation of the procedure of Ellman [9]. After denaturation in 1% SDS, the protein (0.15 mg/ml at pH 8.0) was reacted with 0.5 mM 5,5'-dithiobis-(2-nitrobenzoic acid) as described previously [10,11].

Succinylation of spectrin was accomplished by mixing the protein with a 100-fold molar excess of succinic anhydride (based on a lysine content of approx. 250 residues per dimer of polypeptides 1 and 2 [12]), incubating for 30 min at 25°C while pH was maintained at 8.0, and dialyzing against 50 mM phosphate (pH 8.0).

Amidination of spectrin was carried out using either isethionyl acetimidate or ethyl acetimidate. The typical procedure was as follows. A spectrin solution (0.1—0.2 mg protein/ml) was adjusted to pH 10.0 at 25°C and mixed, at 15-min intervals, with four portions of imidoester, each amounting to a 250-fold excess over lysine residues. The pH was carefully maintained at 10.0 throughout the reaction. The protein was then dialyzed at 4°C against 50 mM phosphate (pH 8.0)/5 mM β-mercaptoethanol, followed by 50 mM phosphate (pH 8.0) alone. A control exposed to pH 10.0 without imidoester was prepared alongside.

Amidination reactions were run at pH 10.0 rather than pH 8.5, which is frequently used for proteins [13], because spectrin undergoes considerable cross-linking at the lower pH. This is evidently due to side reactions involving coupling of *N*-alkyl imidate groups on the protein with amino groups [14]. At pH 10.0 such reactions became relatively insignificant compared to the expected amidine formation.

The extent of NH_2-group modification by succinylation or amidination was determined by reacting spectrin samples (in 50 mM sodium borate, pH 9.1) with fluorescamine, and measuring fluorescence at 475 nm (excitation 390 nm) on an Aminco SPF-125 Fluorimeter. Other details of the analysis were taken from Bohlen et al. [15]. Alternatively, the protein was subjected to SDS gel electrophoresis after reaction with fluorescamine, and the gels were analyzed in a Gilford Fluorescence Gel Scanner (Model 2515), as described by Ragland et al. [16]. The latter procedure had the advantage of showing the loss of NH_2 groups in each spectrin subunit.

Irradiation procedure. Membrane suspensions and spectrin solutions were irradiated at 10°C in a thermostatically controlled 4-place stirrer bath (Yellow Springs Instruments, Model 5301) as described previously [17]. A sharp-cut yellow filter (Corning No. 3484) was used to restrict incident light to wavelengths maximally adsorbed by methylene blue. Light intensity at the sample surface was approx. 200 W/m^2 in all experiments.

Electrophoresis. Conventional SDS-disc gel or SDS-slab gel electrophoresis was carried out according to published procedures [3,7], using 5% polyacrylamide. In some instances, composite gels of 3.5% polyacrylamide/0.4% agarose were used [18]. Unless indicated otherwise, membrane or spectrin samples to be electrophoresed were preincubated for 30 min at 37°C in the presence

of 40 mM dithiothreitol. Detection and quantitation of protein bands were accomplished either by staining with Coomassie blue and scanning for absorbance at 550 nm, or by pretreating samples with fluorescamine and scanning for fluorescence (see above). A planimeter was used for integration of protein peaks.

Ultracentrifugation. Sedimentation velocity runs were carried out with a Spinco Model E analytical ultracentrifuge operated at 60 000 rev./min and 20°C. Solutions of spectrin were concentrated by ultrafiltration to at least 1 mg protein/ml before centrifugation. Schlieren optics were employed, and sedimentation coefficients ($s_{20,w}$) were determined in the conventional manner [19], using published values for partial specific volume and solvent viscosity and density [20,21].

Results

The typical effect of methylene blue-sensitized photo-oxidation on erythrocyte membrane proteins is shown in Fig. 1. With increasing light dosage

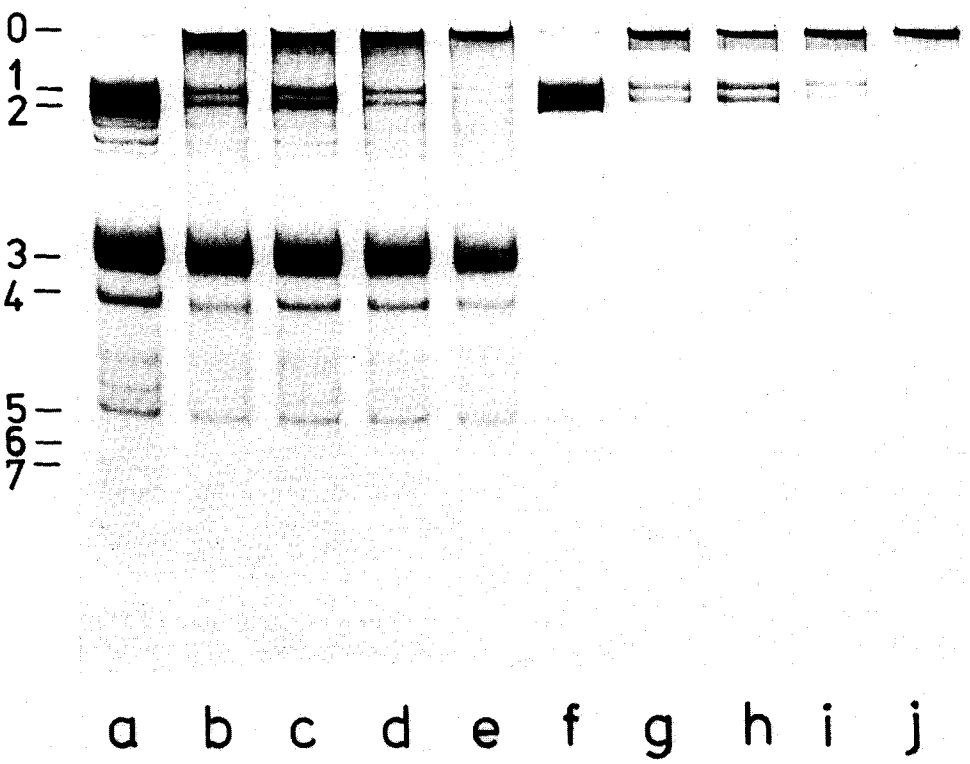

Fig. 1. Photosensitized cross-linking in membranes and in isolated spectrin. Erythrocyte ghosts were washed and resuspended with 50 mM sodium phosphate (pH 8.0) to a protein concentration of 2 mg/ml. The suspension was irradiated in the presence of 10 μM methylene blue for 0 min (a), 15 min (b and c), 30 min (d) and 60 min (e). Spectrin (0.5 mg/ml) was irradiated under the same conditions for 0 min (f), 15 min (g and h), 30 min (i) and 60 min (j). All samples except b and g were treated with 40 mM dithiothreitol before electrophoresis. Protein load per slab gel channel: 18 μg (ghosts); 4.5 μg (spectrin). Bands are numbered according to the nomenclature of Steck [18].

(channels a—e), one notices a gradual decrease in the Coomassie blue staining intensity of certain protein bands (most prominently the spectrin bands), and corresponding accumulation of unresolved, high molecular weight aggregates near the origin. No effect was observed when irradiation was carried out in the absence of methylene blue. The photoaggregation of spectrin is not dependent on its association with intrinsic membrane proteins or lipids, since the isolated protein (containing traces of erythrocyte actin, band 5) is also susceptible to this reaction (channels f—j). Qualitatively, the effects shown here with methylene blue are similar to those described previously with photosensitizers of the tetrapyrrole family [3,5,11,22]. By analogy with results obtained earlier, e.g., persistence of the protoporphyrin-induced photoaggregates in boiling 1% SDS/8 M urea [22], it appears that the methylene blue-induced photoproducts are cross-linked in some manner, rather than associated by unusually stable non-covalent forces.

As a means of showing whether cross-linking can be accounted for by -S-S- bond formation, irradiated samples were treated with dithiothreitol before electrophoresis. A comparison of the 15-min membrane samples in Fig. 1 shows a slight intensification of bands 1 and 2 after (gel c) as compared to before (gel b) reductive treatment. A similar results was obtained with free spectrin (samples g and h). Such partial reversal by reduction gradually diminished with increasing light dose. These results suggest that -S-S- bond formation, exclusive of other types of cross-linking, plays only a minor role in this reaction. It is not known, moreover, whether the limited, apparent -S-S- bridging seen at 15 min (Fig. 1) is due to direct photo-oxidative coupling of SH-groups, or to secondary dark reactions in the modified proteins. In a separate experiment, the question of SH-group involvement was examined by means of chemical modification prior to irradiation. Based on its reactivity with 5,5′-dithiobis(2-nitrobenzoic acid), spectrin was found to contain 35—37 SH groups per heterodimer (460 000 daltons). This value agrees closely with previous values obtained by amino acid analysis [12]. Blocking approx. 85% of these groups with *N*-ethylmaleimide had no effect whatsoever on cross-linking, as measured by the rate of disappearance of bands 1 and 2 on SDS-gel electropherograms (data not shown). These results not only argue against

TABLE I

EFFECT OF IRRADIATION ON SULFHYDRYL AND AMINO GROUPS IN SPECTRIN

Spectrin was irradiated in the presence of 10 μM methylene blue and samples were taken at the indicated times for determination of SH groups. Sulfhydryl content is based on a molecular weight of 460 000 for the spectrin heterodimer [20]. Values shown are averages ± deviations from duplicate experiments. Numbers in parentheses represent remaining groups (per cent). Measurements of NH_2-group reactivity with fluorescamine [15] were also carried out; values are averages ± deviations of duplicate determinations.

Irradiation time (min)	SH groups (mol/mol protein)	NH_2 groups (relative fluorescence)
0	33.9 ± 0.2 (100)	100
20	—	100 ± 2
30	22.4 ± 1.2 (66)	—
40	—	103 ± 3
60	16.7 ± 0.9 (49)	—
90	—	101 ± 9

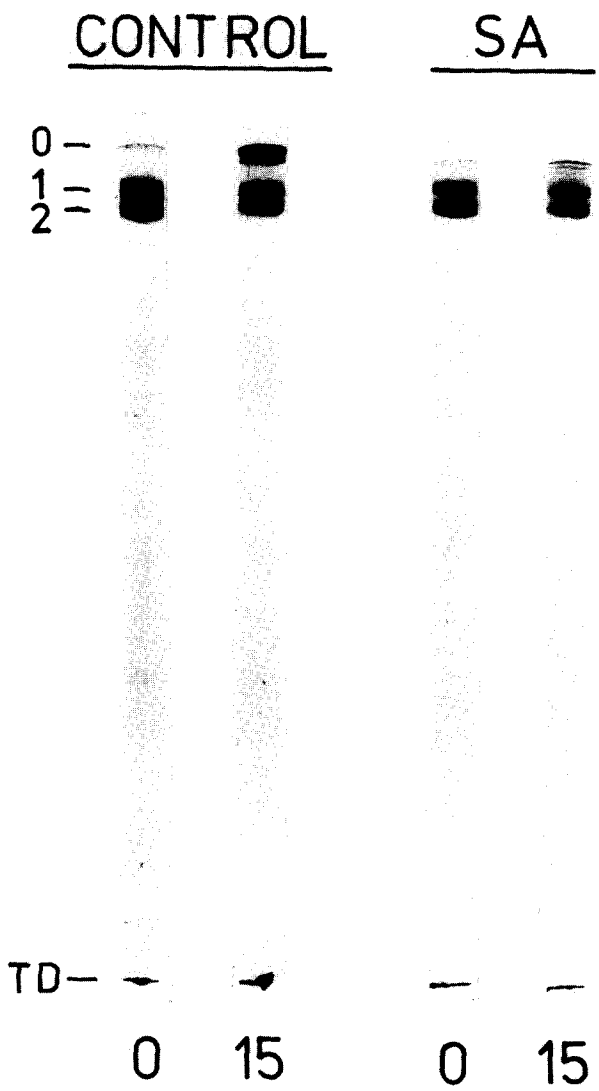

Fig. 2. Effect of succinylation on cross-linking. Succinic anhydride (SA)-treated spectrin (0.5 mg/ml in 50 mM phosphate, pH 8.0) was irradiated in the presence of 10 μM methylene blue for the indicated times (minutes). A non-succinylated control was irradiated alongside. Protein load per gel: 7.5 μg. TD, Pyronin Y tracking dye.

-S-S- bonds, but also against any other type of cross-link involving cysteine residues.

Although SH groups are not implicated in cross-linking, such groups are nevertheless destroyed during photo-oxidation. One sees in Table I that the SH content of spectrin decayed with a half-time of approx. 1 h. Since free cysteine can be converted to cysteic acid by dye-sensitized photo-oxidation [23], this reaction may account for the losses seen here.

In addition to cysteine residues, histidine, methionine, tyrosine and tryptophan residues in proteins are known to be susceptible to photodynamic action

[23]. It is natural to assume that photoproducts of some of the latter four types of residue might react with certain acceptor groups to give stable cross-links such as described here. Previous studies carried out with free amino acids have provided some clues as to the possible participants in such reactions. For example, Tomita et al. [24] showed that photo-oxidation of histidine or *N*-benzoylhistidine gives rise to a complex mixture of intermediates, some of which appeared to be dimers generated by nucleophilic attack of residual starting material on oxidized imidazole groups. Recent studies by Straight and Spikes [25] showed that methylene blue-sensitized photo-oxidation of side chain groups in histidine, methionine, tyrosine and tryptophan is accompanied by a loss of α-amino groups, as measured by fluorescamine reactivity. Furthermore, photo-oxidation of imidazole in the presence of a non-photo-oxidizable amino acid such as glycine also resulted in an initial rapid disappearance of the NH_2 group, suggesting that the latter had somehow coupled with imidazole photoproduct(s). These findings raised the possibility that membrane protein cross-linking might be explained along the same lines. To examine this question, we specifically focused our attention on the possible role of NH_2 groups in the reaction.

As shown in Table I, photo-oxidation of spectrin under conditions that cause a large decrease in SH titer and extensive cross-linking (Fig. 1) has no significant effect on NH_2 groups. Although this result would seemingly rule out NH_2 involvement first-hand, it is conceivable that any losses that do occur are too small to be detected. Since spectrin contains approx. 125 lysine residues per subunit [12], a single cross-link involving an ϵ-NH_2 group would decrease the NH_2 content by less than 1%.

As a further test of NH_2-group reactivity, chemical modification was carried out prior to irradiation. Preliminary experiments were performed with succinic anhydride-treated spectrin (Fig. 2). It can be seen that the succinylated protein (in which no residual NH_2 groups could be detected) was almost completely protected against cross-linking, as compared to a control exposed to the same light dose. Similar results were obtained with other photosensitizers (e.g., protoporphyrin IX, rose bengal), indicating that this result is not unique with methylene blue.

TABLE II

SEDIMENTATION COEFFICIENTS OF NATIVE AND CHEMICALLY MODIFIED SPECTRIN

Succinylated spectrin (the same materials described in Fig. 2) and an unmodified control prepared alongside were subjected to analytical ultracentrifugation (Expt. 1). A separate preparation was analyzed after reaction with ethyl acetimidate (Expt. 2). The protein concentration in each case was 1.2 mg/ml in a solvent consisting of 50 mM sodium phosphate (pH 8.0), 50 mM NaCl, 0.1 mM EDTA and 6 mM β-mercaptoethanol.

	Spectrin sample	Number of schlieren peaks resolved	$s_{20,w}$ (S)
Expt. 1	Control	1	9.1
	Succinylated	1	4.1
Expt. 2	Control	2	9.0; 12.2
	Amidinated	2	8.7; 11.9

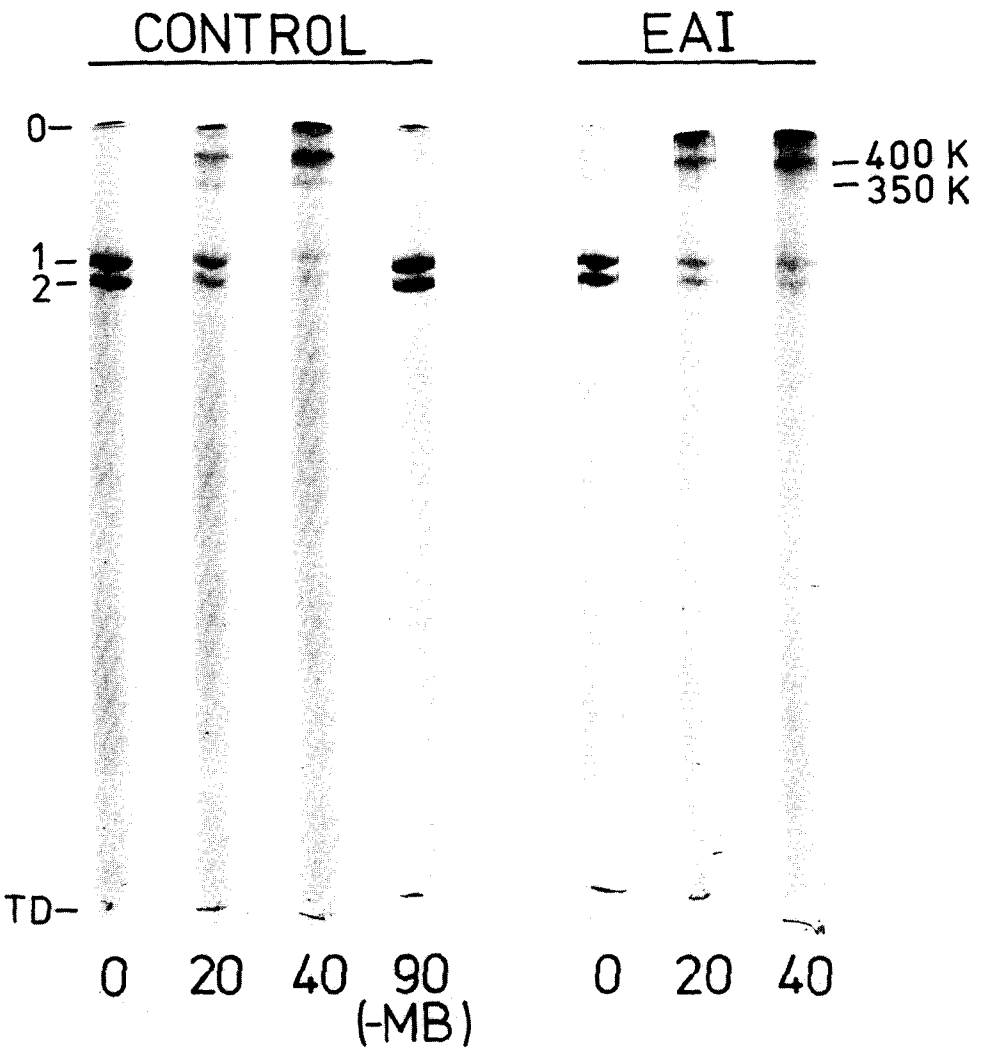

Fig. 3. Cross-linking of amidinated spectrin. Ethyl acetimidate (EAI)-treated spectrin (0.15 mg/ml in 50 mM phosphate, pH 8.0) was irradiated for 0, 20 and 40 min in the presence of 10 μM methylene blue, then analyzed by SDS-polyacrylamide/agarose gel electrophoresis. The non-amidinated control was irradiated in the presence (0, 20, 40 min) and absence (90 min) of methylene blue (MB). Protein load per gel: 4.4 μg. Protein stain: Coomassie blue. TD, Pyronin Y tracking dye.

Although succinylation blocks primary amino groups, it may also cause polypeptides to unfold and dissociate because of charge repulsion. The extent of such secondary effects is succinylated spectrin was assessed by analytical ultracentrifugation (Table II, Expt. 1). The sedimentation coefficient of the control protein is 9.1 S, which agrees closely with previous values reported for the spectrin heterodimer [20,26]. On the other hand, the $s_{20,w}$ value of 4.1 S for succinylated spectrin indicates that it has undergone extensive conformational changes, and presumably exists in the form of monomers [20]. Thus, inhibition of photosensitized cross-linking in the succinylated protein may have

been due to these secondary factors. This possibility was checked by comparing succinylation with an alternative method such as amidination, which is reported to have only minimal perturbing effects on protein conformation [27]. Under the modification conditions described (see Materials and Methods), the extent of conversion of NH_2 groups to amidino groups was routinely 95% or greater, as determined by fluorescamine assay. The sedimentation characteristics of peramidinated spectrin and its control are shown in Table II (Expt. 2). In each case, two schlieren peaks were resolved, a major one at approx. 9 S and a minor one at 12 S. The latter evidently represents a small amount of tetramer, which is occasionally seen in spectrin preparations [20]. The fact that the $s_{20,w}$ values for the modified protein differ from those of the control by less than 5% suggests that protein structure was largely preserved after amidination.

The effect of photo-oxidation on amidinated spectrin is shown in Fig. 3. It is apparent that cross-linking still occurs after modification, and that the time course of the reaction is similar to that of a control irradiated simultaneously. Examination of the polyacrylamide/agarose composite gels used in this experiment reveals a partial resolution of the photoaggregated protein into at least two intermediates (approx. 350 000 and 400 000 daltons). These intermediates are presumed to be cross-linked dimers that have low apparent molecular weights because of shape anomalies and/or diminished SDS binding [28]. A quantitative comparison of spectrin losses due to cross-linking was accomplished by scanning the gels in Fig. 3 and integrating over bands 1 and 2. As shown in Fig. 4, spectrin loss followed first-order kinetics down to less than

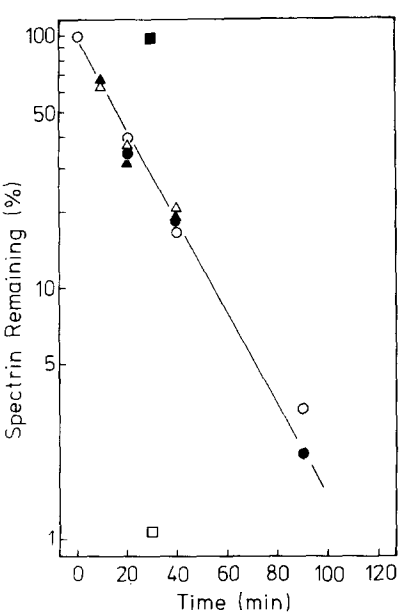

Fig. 4. Time course of spectrin loss before (○) and after (●) amidination. Gel electropherograms from the experiment shown in Fig. 3 were scanned and integrated to quantitative protein loss. A comparison is made with the effect of 5 mM glutaraldehyde (30 min exposure at 25°C) on control (□) and amidinated (■) spectrin. Also shown is the loss of spectrin in the absence (△) and presence (▲) of ethylamine (5 mM) during irradiation (see Fig. 3 for photoreaction conditions).

5% spectrin remaining. Significantly, the peramidinated protein disappeared at exactly the same rate as the unmodified control. Similar results were obtained using protoporphyrin IX as the photosensitizer (data not shown). It should be noted that fluorescamine-reactive NH_2 groups did not reappear during the course of irradiation, thereby ruling out any possible large-scale removal of amidino groups or generation of new NH_2 groups. Although amidination of NH_2 groups had no effect on photosensitized cross-linking, it did, by way of contrast, inhibit glutaraldehyde-mediated cross-linking almost completely (Fig. 4). This result is not unexpected, since only the NH_2 groups of erythrocyte membrane proteins are known to be susceptible to glutaraldehyde bridging [18]. As an additional check on possible NH_2-group involvement in photosensitized cross-linking, photoreactions were carried out in the presence of low molecular weight amines as possible competitors. Thus, with ethylamine (added in 50-fold molar excess over total lysine groups in spectrin), there was no detectable inhibition of cross-linking (Fig. 4). Similarly, addition of free lysine was without effect (data not shown).

Discussion

A mechanism for photosensitized cross-linking of erythrocyte membrane proteins has recently been proposed by Dubbelman et al. [29]. In this scheme, reaction products of photo-oxidizable residues, primarily those of histidine, undergo nucleophilic attack by NH_2 groups to give stable cross-links. These secondary reactions are presumed to be light-independent. This proposal was based partially on model studies with free amino acids. For example, protoporphyrin-sensitized photo-oxidation of histidine in the presence of a radioactively labeled non-photo-oxidizable amino acid such as glycine resulted in the formation of new labeled components (presumably products of coupling reactions), as visualized by thin-layer chromatography and autoradiography. Dubbelman et al. [29] reinforced their contention that NH_2 groups play a crucial role in cross-linking by showing that protoporphyrin-sensitized photopolymerization of spectrin (in the membrane-bound as well as membrane-free state) is greatly inhibited by prior succinylation of the protein. A similar observation was made in the present study, using methylene blue as the sensitizing agent. Hydrodynamic measurements revealed, however, that succinylation of spectrin not only blocks NH_2 groups, but causes extensive conformational changes such that any conclusions regarding subsequent reactivity of NH_2 groups per se are severely clouded. As a further illustration of this point, 2,3-dimethylmaleic anhydride is known to unfold and elute peripheral proteins from erythrocyte ghosts [30], and this appears to be the explanation for its protective action against bilirubin-photosensitized cross-linking [11]. The above ambiguities were eliminated in the present work by employing a chemical modification that causes relatively little change in protein conformation. Thus, amidination left spectrin intact, and had no effect whatsoever on light-induced cross-linking. In addition, attempts to inhibit competitively this reaction by irradiating in the presence of exogenous primary amines gave negative results. Contrary to the previous proposal [29], therefore, these findings suggest that NH_2 groups do not play a central role in the cross-linking reaction.

Certain other possibilities relating to the cross-linking mechanism have also been ruled out. For example, even though the dye-sensitized photo-oxidation of free cysteine to cystine has been demonstrated [31], the predominant bonds formed in the present system are not -S-S- bonds. Furthermore, the possibility that bridging occurs via methylene blue in its excited state, or some photo-product of methylene blue, is unlikely, since qualitatively the same reaction is observed with several different photosensitizers, e.g., protoporphyrin, bilirubin, rose bengal [3,11,22].

While definitive evidence concerning the identity of cross-linkable residues is not yet available, recent preliminary experiments in this laboratory have implicated tyrosines. Treatment of isolated spectrin with *N*-acetylimidazole under conditions that produce *O*-acetyltyrosine residues in proteins [32] caused a substantial inhibition of methylene blue-sensitized cross-linking, similar to that seen after succinylation (cf. Fig. 2). Acetylation of NH_2 groups in this reaction was relatively modest (less than 15% reacted), thereby reducing (but not eliminating) uncertainties about conformational changes. If tyrosine phenolic groups prove to be involved, they apparently do not cross-link with one another, since Dubbelman et al. [29] did not detect a characteristic dityrosine fluorescence in photo-oxidized spectrin.

Additional studies aimed at elucidating the cross-linking mechanism are currently under way in this laboratory.

Acknowledgements

This work was supported by Grant HD-07753 from the National Institutes of Health. The author is grateful to Drs. E.A. Dratz and J.D. Spikes for helpful discussions. The excellent technical assistance of Mrs. Carol Svatek is also appreciated.

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