Photoactivated Cross-linking of Proteins within the Erythrocyte Membrane Core*

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We describe the reactions of three lipophilic, photoactivated cross-linking reagents, 1,5-diazidonapthalene, 4,4'-diazidobiphenyl, and the reversible 4,4'-dithiobisphenylazide, with erythrocyte membranes. Cross-linking occurs only upon photoactivation. At pH 7 to 8, only spectrin components are cross-linked by these reagents. At pH 5.0 to 5.5 several additional membrane proteins including the major “integral” membrane proteins are also cross-linked, despite equivalent binding of the cross-linkers at neutral and acid pH. The cross-linking rates of various membrane proteins at pH 5.0 to 5.5 depend distinctly upon duration of photoactivation. Bidimensional electrophoresis of membrane proteins after cross-linking with the reversible cross-linker, 4,4'-dithiobisphenylazide, has allowed for the identification of homopolymeric products of cross-linking (e.g. dimers and tetramers of Band 3) and heterocomplexes (spectrin plus other membrane proteins). The data suggest that at reduced pH, cross-linking can proceed not only at the membrane surface but also in the membrane core.

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MATERIALS AND METHODS

Erythrocyte membrane vesicles were isolated from freshly drawn, heparin-treated blood according to the standard procedure of Fairbanks et al. (11). Prior to irradiation, the membranes were washed twice in the desired buffer. Membrane protein was determined as in Ref. 11.

Diazidonapthalene and diazidobiphenyl were synthesized from their respective amines (Alrich) by the established procedures (Method A) of Smith and Brown (12, 13). 4,4'-dithiobisphenylazide was also synthesized by these procedures from 4-aminothiophenol (Alrich), except that the Norit extraction step was deleted. Subsequent to azide formation, disulfide formation was accomplished according to Ref. 14. Complete conversion to disulfide was indicated by the absence of reaction with Ellman’s reagent (15). To obtain radioactively labeled 4,4'-diazidobiphenyl [14C]benzidine (New England Nuclear, 30 mCi/mmol) was included in the synthesis. Radioactivity was counted in a Packard scintillation counter with Bray’s scintillation mixture (16).

Unidimensional dodecyl sulfate electrophoresis was performed as described in Ref. 11 with 5.6% acrylamide in 3-mm inner diameter gel tubes and in the absence of mercaptoethanol. The gels were stained with Coomassie blue for protein and by the Schiff periodate method for carbohydrates (11). The protein bands were enumerated as in Ref. 17. No differences in staining patterns were observed in nonilluminated controls at pH 5.0 or 8.0.

In bidimensional studies, electrophoresis in the second dimension was in 4 to 30% acrylamide gradient slab gels (Pharmacia). The first dimension gel was removed from its tube and dialyzed for 30 min against the electrophoresis buffer containing 0.01 M 2-mercaptoethanol. The gel was then placed upon the slab at its low acrylamide end and perpendicular to the direction of the acrylamide gradient.
trophoresis in the second dimension was at 25 mA for 5 h in a Pharmacia gel apparatus GE-4.

The illumination apparatus consisted of a Perkin-Elmer MPF-3 spectrofluorometer with a magnetic stirrer mounted overhead to provide continuous mixing during irradiation. Temperature was maintained at 37° by means of a thermostated cuvette holder connected to a water circulator. Typically, membranes (1.0 mg/ml; 1.0 ml) were pre-equilibrated for 5 min in the dark at 37° with cross-linking reagent added as ethanolic solutions to final concentrations of $10^{-5}$ to $10^{-4}$ M (1% ethanol). At selected times of illumination, 50- to 100-μl aliquots were removed and immediately added to test tubes containing 5 to 10 μl of 20% sodium dodecyl sulfate. Two types of control were used: nonilluminated samples and samples exposed to ethanol alone. For experiments in which oxygen was excluded, samples were irradiated in a Thunberg fluorometer cuvette after purging for 1 h with nitrogen cleansed of traces of oxygen by prior passage through alkaline pyrogallol (18). Dithiothreitol at 0.1 mM was incorporated into the reaction mixtures to further assure reducing conditions. Irradiation was at 520 nm, with 40-nm slit width.

Chloroform-methanol extraction of membranes was as in Ref. 19.

**RESULTS**

Dodecyl sulfate polyacrylamide gel electrophoresis (Fig. 1) shows that when erythrocyte membranes equilibrated with 1,5-diazidonaphthalene or 4,4'-diazidobiphenyl at concentrations of $10^{-9}$ to $10^{-8}$ M and pH 7.5 to 8.0 are irradiated for intervals of 30 min or less, protein cross-linking is limited to the spectrin region (Bands 1 and 2). However, irradiation performed at pH 5.0 to 5.5 under otherwise identical conditions produces extensive cross-linking of several additional protein components by the reduction of staining intensity in the region of Bands 3 and 4 and accumulation of material at the top of the gel.

We have also monitored cross-linking of glycoproteins using dodecyl sulfate polyacrylamide gel electrophoresis and periodic acid-Schiff staining (11). We find no cross-linking at pH 7.0 to 8.0 and no more than 10% loss of staining in the region of the major periodic acid-staining glycoprotein I after irradiation for 20 min at pH 5.0.

Further characterization of the cross-linking reaction revealed that the cross-linking requires the presence of reagent, but not oxygen, indicating that the cross-linking reaction involves the covalent participation of the reagents and is not a photosensitization reaction. Moreover, an intact membrane structure is prerequisite since no cross-linking is observed when the irradiation is performed in the presence of 0.1% dodecyl sulfate.

The reversible cross-linker, 4,4'-dithiobisphenylazide, behaves as do 1,5-diazidonaphthalene and 4,4'-diazidobiphenyl with respect to irradiation conditions and pH. In this case, however, the reacting membrane proteins can be specified by bidimensional dodecyl sulfate-polyacrylamide gel electrophoresis: cross-linking is reversed by reduction of the S-S bond of 4,4'-dithiobisphenylazide prior to electrophoresis in the second dimension. The results (Fig. 2) show that after reaction at pH 5.0 Band 3 exists in several polymeric species including dimer, tetramer, and possibly higher molecular weight entities. Components 4.1, 4.2, 4.5, and 5 also form heterogeneous complexes of high molecular weight at low pH, but we cannot distinguish well defined intermediate states such as dimers or tetramers. It is possible that heteropolymeric species including spectrin are formed, as suggested by Wang and Richards (6, 7). At the top left corner of the bidimensional slab gel there is Coomasie blue staining material of very high molecular weight which is not reduced in size even at high concentrations of 2-mercaptoethanol (0.2 M). We suspect that these reaction products result from cross-linking induced by triplet nitrene hydrogen abstraction processes rather than singlet nitrene insertion reaction (20). Whether different intramembrane environments influence the intersystem crossing necessary to generate the triplet nitrene, thus selectively cross-linking different proteins, cannot be ascertained at present.

We have followed the cross-linking of individual protein components as a function of illumination time by unidimensional electrophoresis. Although this method cannot distinguish whether cross-linking produces hetero- or homopolymeric species, gradient gels have sufficient fractionating power to allow one to monitor the rate of disappearance of major membrane protein components. The results of such a

![Fig. 1. Electrophoresis before and after irradiation.](image)

**Fig. 1.** Electrophoresis before and after irradiation. Irradiation was for 10 min in the presence of $10^{-9}$ to $10^{-8}$ M diazidobiphenyl. 70 μg of protein were applied to each gel. ---, 0 min, 5 mM sodium citrate, pH 5.0; ---, 10 min, 5 mM sodium phosphate, pH 5.0; ---, 10 min, 5 mM sodium citrate, pH 8.0. The 0-min control samples at pH 5.0 and pH 8.0 demonstrated identical staining patterns.

![Fig. 2. Bidimensional electrophoresis after irradiation at pH 5.0 with 4,4'-dithiobisphenylazide.](image)

**Fig. 2.** Bidimensional electrophoresis after irradiation at pH 5.0 with 4,4'-dithiobisphenylazide. Illumination for 2 min at $10^{-5}$ M 4,4'-dithiobisphenylazide. 120 μg of protein were applied to the first dimension gel. Conditions for bidimensional electrophoresis are described under "Materials and Methods." The arrows indicate the direction of electrophoresis for the first and second dimensions. The arrow for the second dimension also marks the top of the first dimension gel.
rate experiment at pH 5.0 are summarized in Fig. 3 and demonstrate that the cross-linking reactions of several different membrane protein components proceed at different rates. Spectrin components 1 and 2, and Band 5 are cross-linked most rapidly, whereas Band 6 is not cross-linked at all during a 20 min illumination. Intermediate reaction rates are observed for Band 3 and components of Bands 4.1, 4.2, and 4.5. It is clear, however, from Fig. 2 that not all of the protein components of the diffuse Band 4.5 participate in cross-linking. Further interpretation of these data in terms of absolute rates is complicated by positive contributions of Coomassie blue staining by cross-linked polymers of low molecular weight proteins to the staining in regions of higher molecular weight entities.

To determine whether the cross-linking reactions of different proteins can be distinguished according to the pH at which 50% cross-linking occurs, we have examined the pH dependency of cross-linking for components 1, 2, 3, and 4.1 and 4.2 (Fig 4). These results indicate that component 3 and spectrin (1, 2) are cross-linked in a reaction with a midpoint pH of 5.9 in contrast to the cross-linking of 4.1 and 4.2 which is characterized by a midpoint pH of 5.6. The cross-linking reaction of spectrin (and perhaps 4.1 and 4.2) also shows a high degree of cooperativity not seen with component 3. This apparent absence of cooperativity in the component 3 cross-linking reaction may stem from the known heterogeneous composition of Band 3 and/or heterogeneity in the cross-linking reaction, e.g. formation of homopolymers (dimer and tetramer) versus heteropolymers (component 3 + spectrin).

It appears from the data in Figs. 1 to 4 that a reduction in pH results in extensive cross-linking of erythrocyte membrane protein. However, it is also possible that the pH shift induces a change in the distribution of our cross-linking reagents in the membrane. To test for this we have examined the distribution of [14C]diazidobiphenyl before and after illumination at pH 5.0 and pH 7.5. The results in Table I indicate that the change in pH does not alter the amount of cross-linker that has reacted covalently with protein.

### DISCUSSION

In the present report we have examined proximity relationships within the erythrocyte membrane core by employing lipophilic d&ides to monitor alterations in protein-protein association induced with changes in bulk pH. Our results

**Fig. 4.** The effect of pH on the cross-linking reactions of components 1 and 2 (spectrin), 3, and 4.1 and 4.2. Conditions of illumination: protein, 1.0 mg/ml; 4,4'-dithiobisphenylazide, $2 \times 10^{-5}$ M; time, 10 min. The results represent the average from two gels. The absolute amount of absorbance was determined by the weighing procedure specified in the legend to Fig. 3. Buffers in this particular experiment were 5 mM citrate from pH 5.3 to 6.0 and 5 mM phosphate at pH >6.0. In other experiments similar results were obtained with phosphate buffers at pH b.0 to 7.0. 0——0, Spectrin; △——△, Band 3; □——□, Band 4.1 and 4.2.

**Table I**

<table>
<thead>
<tr>
<th>pH</th>
<th>Sample</th>
<th>Total Cpm</th>
<th>Organic</th>
<th>Aqueous</th>
</tr>
</thead>
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<tr>
<td>5.0</td>
<td>$t = 0$ min</td>
<td>99,500 (100%)</td>
<td>100,720 (103%)</td>
<td>2,838 (3%)</td>
</tr>
<tr>
<td></td>
<td>$t = 30$ min</td>
<td>31,209 (31%)</td>
<td>70,924 (71%)</td>
<td></td>
</tr>
<tr>
<td>8.0</td>
<td>$t = 0$ min</td>
<td>102,407 (100%)</td>
<td>107,184 (105%)</td>
<td>4,567 (4%)</td>
</tr>
<tr>
<td></td>
<td>$t = 30$ min</td>
<td>32,368 (32%)</td>
<td>73,946 (72%)</td>
<td></td>
</tr>
</tbody>
</table>

*Effect of pH on [14C]diazidobiphenyl distribution*

Illumination conditions are as described in the legend of Fig. 1. The membranes were extracted as described under "Materials and Methods."
indicate that a low pH allows cross-linking of several proteins that are resistant at pH 7.5 to 8.0. This effect of pH is not due to an effect of pH on the degree of covalent, photoactivated binding of cross-linker since irradiation produces equivalent incorporation at the two pH values of covalently linked [14C]diazidobiphenyl into protein (Table 1). Thus, although total membrane protein reacts with equal facility at pH 5.0 to 8.0, the proteins undergo substantial rearrangement within the membrane upon altering pH.

One explanation for our results is found in the various membrane models requiring a cytoplasmic protein framework for maintenance of membrane structure (21). It is possible, for example, that the nonaggregated arrangement of intramembranous particles within the plane of the erythrocyte membrane is stabilized by a spectrin framework. At low pH these structural restraints are removed with resultant particle aggregation (1). However, this possibility cannot fully explain the effect of pH on the degree of covalent, photoactivated cross-linking. Indeed electrophoretic analysis after exposure to low pH, under our conditions, shows no spectrin extraction. Second, our data (Fig. 2) are not in complete accordance with models postulating a spectrin-maintained segregation of membrane proteins. According to such models, the contacts between spectrin and these penetrating proteins should be maximal in the nonaggregated state. However, the data in Fig. 2 indicate that at low pH, where protein cross-linking is enhanced, products of the cross-linking reaction include not only homopolymers of Band 3 but heteropolymers of several protein components that form complexes with spectrin. Similar conclusions concerning the composition of high molecular weight complexes formed with imidates have been reached by Wang and Richards (6).

Another explanation for our results follows from previous proposals that membranes consist of restrained two-dimensional lipid-protein domains. The stability of these domains depends on certain intrinsic forces which have several properties, amongst which is a high degree of pH sensitivity. Evidence from ESR spectroscopy indicates that a reduction in pH not only increases erythrocyte membrane lipid mobility but in the process substantially alters protein-lipid interaction within the membrane core (22). Also, pH reduction markedly increases the accessibility of membrane protein fluorophores to lipid-soluble fluorescence quenchers (2). Finally, Raman spectroscopic evidence suggests the possibility that the lipid-protein interfaces constitute a distinct phase (as opposed to pure lipid or pure protein) and that state transitions can be induced in this phase by a reduction in bulk pH (3). We note that the midpoint pH 6.1 of the transition observed by Raman spectroscopy is similar to the pH 5.9 at which 50% cross-linking of Band 3 and spectrin occurred.

From the above evidence we argue that our increased cross-linking results from a bulk pH-induced relaxation of “normal” protein-lipid interaction which at neutral pH maintains the segregated state of membrane proteins. The high H+ concentration at the membrane surface may register within the membrane core by an “unfolding” of surface proteins (e.g., spectrin) or of the surface extremity of a penetrating protein. This conformational change is transmitted into the membrane interior via the penetrating protein components, resulting in altered lipid-protein contacts. Alternatively, it is conceivable that the effects of pH on these lipid-protein interactions can be explained by enhanced charge effects in regions of low dielectric constant such as found in the membrane core.

Our interpretations are compatible with previous experiments with glutaraldehyde and imidates where cross-linking of several proteins was observed at neutral pH (6, 7) if it is appreciated that the lipophilic arylazides localize primarily in apolar membrane regions, while the other cross-linkers react at the cell surface. Considerable evidence indicates that the lateral mobility of proteins within the erythrocyte membrane core is severely restricted (21-23). In contrast, surface proteins or surface portions of proteins are expected to show greater structural and translational flexibility, allowing for greater and more varied protein-protein contacts.

The fact that spectrin cross-linked at both neutral and low pH with our lipophilic probes indicates that our probes do not localize exclusively within the membrane interior. It is possible, however, that spectrin (as many other proteins that participate in monomer-polymer equilibria) has hydrophobic face(s) at points of monomer contact and that the lipophilic probes react at such sites.

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REFERENCES
Photoactivated cross-linking of proteins within the erythrocyte membrane core.
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