A New Radioactive Cross-linking Reagent for Studying the Interactions of Proteins

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We have developed a photoactivatable, heterobifunctional, reversible, radioactively labeled, chemical cross-linking reagent, 3-{(2-nitro-4-azidophenyl)-2-aminoethylthio]-N-succinimidyl propionate, for studying the interactions of proteins in situ. When reacted in the dark with a purified protein, it forms a covalent derivative which can be purified and reconstituted into biological systems. This derivative will form cross-links to neighboring macromolecules only upon photolysis; reduction cleaves the cross-link and transfers the radiolabel to the second molecule, which can then be identified by standard techniques. We have tested the cross-linker using the binding of gelatin to fibronectin. The cross-linker gives the proper chemical behavior under biological conditions, reacts with high yield and with a very low level of nonspecific cross-linking, and can be used to identify protein-protein and other interactions at the cell surface and elsewhere. The advantages, limitations and possible uses for this reagent are discussed.

The structures, organization and functions of cytoskeletal filaments, membranes, and extracellular matrices depend on specific interactions between many macromolecules. A major problem which faces cell biologists is to identify these specific interactions. One approach is to isolate macromolecules and examine their interactions in vitro, but this approach is limited by the difficulty of extrapolating results to the situation in living cells. The in vitro approach needs to be supplemented with methods for analyzing protein-protein interactions in situ.

Interactions within assemblies of proteins have been difficult to analyze in situ because of their complexity. Chemical cross-linking reagents have been applied in several complex systems (see Ref. 1 for review), but cross-linking reagents also have limitations. Simple bifunctional cross-linkers produce very complex cross-linking patterns which are difficult to analyze. Heterobifunctional cross-linking reagents lead to some simplification; in principle, one can limit the cross-linking to the neighbors of a single protein, which is covalently coupled to cross-linker and then introduced into the biological system (1-3).

A good example of the problems involved in the analysis of protein-protein interactions is the glycoprotein fibronectin. Fibronectin is a major constituent of connective tissue in vitro, and is involved in the adhesion of cells to substrata in cell culture (4-9). Immunofluorescence and electron microscopic studies show that fibronectin forms extracellular fibrils whose arrangement is related to that of the microfilament system inside the cells (10-14).

How does fibronectin participate in these interactions between cells and extracellular substrata? Interactions between purified fibronectin and other macromolecules have been studied extensively in vitro. It has been shown to bind specifically to other molecules of fibronectin, to gelatin, collagen, fibrin, heparin, and other glycosaminoglycans, and to actin and DNA (4-9).

How relevant are these interactions for the functions of fibronectin in situ? Using symmetrical bifunctional cross-linkers, it has been shown that fibronectin can be cross-linked into large aggregates (15). More informative results were obtained when heterobifunctional cross-linking reagents were attached monofunctionally to fibronectin before it was added back to cells (16). These reagents cross-linked the added fibronectin to proteoglycans and to other molecules of fibroin (17). These results are consistent with the in vitro studies but leave important questions unanswered. The cross-linking reagents were not sensitive enough to detect protein-protein contacts which, though functionally important, are likely to comprise only a small fraction of the total interactions. In particular, the membrane-binding site for fibronectin remains unidentified.

In this paper, we report the development of a radiolabeled heterobifunctional, photoactivatable, cleavable, cross-linking reagent which should greatly extend the sensitivity of cross-linking studies. This reagent may be useful for investigating protein-protein interactions in many systems, such as the fibronectin cell system discussed above.

EXPERIMENTAL PROCEDURES

Materials—[35S]-labeled cystamine (1.7 Ci/mmol) was provided by Dr. Mark Staples of New England Nuclear. FNPA† and DTSP were from Pierce. 85% m-chloroperoxybenzoic acid, cystamine-HCl, and triethylamine were from Aldrich. Triethylamine was treated with triethylamine to remove low levels of traces of acrylamide. These reagents cross-linked the added fibronectin to proteoglycans and to other molecules of fibronectin (18). These results are consistent with the in vitro studies but leave important questions unanswered. The cross-linking reagents were not sensitive enough to detect protein-protein contacts which, though functionally important, are likely to comprise only a small fraction of the total interactions. In particular, the membrane-binding site for fibronectin remains unidentified.

In this paper, we report the development of a radiolabeled heterobifunctional, photoactivatable, cleavable, cross-linking reagent which should greatly extend the sensitivity of cross-linking studies. This reagent may be useful for investigating protein-protein interactions in many systems, such as the fibronectin cell system discussed above.

†The abbreviations used are: FNPA, 4-fluoro-3-nitrophénylazide; BSA, bovine serum albumin; DTSP, dithio-bis-(sucinimidyl propionate); DTSPO, dithio-bis-(sucinimidyl propionate)-S,S-dioxide; HSA, human serum albumin; NAC, N-acetyl (2-nitro-4-azidophenyl)-cystamine; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; XL, 3-[2-nitro-4-azidophenyl]-2-aminoethylthio]-N-succinimidyl propionate.

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form were treated with activated alumina and dimethylformamide with silica gel immediately before use.

Gelatin was prepared by boiling collagen from Vitrogen Co. for 5 min. Fibronectin was isolated from fresh human plasma on gelatin-Sepharose CL-4B as described (17, 18). Human IgG was isolated from fresh human plasma on protein A-Sepharose.

Synthesis of NFC-Ac was prepared by a modification of the procedure of Huang and Richards (19). 10.0 mcCi of Na14C-labeled cysteine at 250 mcCi/mmol (40.0 mcCi) was dissolved in 0.30 ml of methanol, and 40 mg of FNPA (215 mcCi) and 35 μl of triethylamine were added. After 48 h in the dark at 35 °C, the dark red precipitate was collected by centrifugation, washed with methanol, and used immediately. The precipitate contained the main product (>90%) which co-migrated with unlabeled NFC on thin layer chromatography on silica gel with ethyl acetate and benzene. The yield was 71% by radioactivity.

Synthesis of DTSPO—0.91 g of 80% m-chloroperbenzoic acid in 50 ml of chloroform was added dropwise over 1 h to a solution of 0.97 g of DTSP (2.32 mmol) in 350 ml of chloroform stirring under nitrogen. The reaction was stirred for 4 h on ice, then for 1 h at room temperature. The solvent was removed by rotary evaporation and the residue was washed with benzene. The crude product was extracted with 60 ml of dioxane and precipitated with a minimum amount of hexane. The product was purified as a single spot on thin layer chromatography in two solvent systems. In methylene chloride:acetone:formic acid (100:25:1), RF = 0.30 and in ethyl acetate, RF = 0.50. IR peaks characteristic of the succinimide ester appeared at 1810, 1780, and 1860 cm⁻¹. Structure 1 shows the NMR assignments for DTSPO.

Synthesis of XL—Reactions were kept in the dark as much as possible, and all manipulations were carried out in dim light. 6.9 mg of diethiothreitol (43 μmol) and 10 μl of triethylamine were added to 7.06 mcCi of NAC at 250 mcCi/mmol (28 mcCi) in 1.0 ml of chloroform. After 1 h at room temperature, 40 μl of DTSP (91 μmol) was added, and the reaction was stirred for 20 min during which time all the DTSPS dissolved. The solvent was evaporated under a stream of nitrogen and the residue was extracted with 2 ml of toluene. This solution was applied to a silicic acid column (5 cm × 1 cm) on a Sephadex G-25 column equilibrated with PBS. The protein absorbance was measured continuously through the near UV. Photolysis was carried out for 10 min at 360 nm in the dark. The protein derivative was separated from unreacted XL by centrifugation, washed with methanol, and used immediately. The product was eluted with chloroform which had been bubbled with nitrogen. The attached cross-linker residue is 15-16 Å long. Upon reduction, the disulfide bond is cleaved, and the radiolabel is left attached to the second reaction site, as shown in Fig. 2.

The Succinimide Ester readily reacts with amino groups on proteins under mild conditions to form a covalent derivative. The attached cross-linker residue is 15–16 Å long. Upon photolysis, the phenylazide is converted to a highly reactive nitrene which can react with many groups on proteins to form a covalent bond at a second site. Upon reduction, the disulfide bond is cleaved, and the radiolabel is left attached to the second reaction site, as shown in Fig. 2.

**RESULTS**

**Cross-linking Scheme**—The synthetic scheme and the structure of the cross-linking reagent XL are shown in Fig. 1.

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**Cross-linking Fibronecin-XL to Gelatin-Sepharose**—To determine if the cross-linking reagent behaves as predicted in the presence of protein, we studied its behavior in a simple model system. Gelatin coupled to Sepharose CL-4B binds...
fibronectin with high efficiency and a defined stoichiometry (4–9). This provides a convenient means for determining the location of the radiolabel after photolysis and reduction.

Fibronectin labeled with approximately 10 cross-linkers/protein molecule was bound to gelatin-Sepharose and then photolyzed, reduced, and boiled in SDS. Control samples remained without the various treatments. Samples were washed to remove unbound radiolabel and the radioactivity bound to the beads was counted.

The results of two such experiments show that before photolysis only 10–17% of the radioactivity remained bound after denaturation in SDS (Table I). After photolysis, SDS-resistant radioactivity increased to 46–54% (Table I, line 6). Taking the level before photolysis as background, these data indicate that 36–41% of the fibronectin became covalently attached to the gelatin-Sepharose beads via the cross-linker. Similarly, the radioactivity resistant to dithiothreitol increased from 3–5% to 29–43% after photolysis, indicating that a minimum of 24–40% of the cross-linker reacted to form a covalent bond through the azide end (Table I, line 7). After treatment with dithiothreitol and SDS, the radioactivity bound to the beads increased from 6–8% before photolysis to 15–26% after photolysis. This indicates that 10–18% of the cross-linker reacted with the gelatin-Sepharose (Table I, line 8). Note that since there is initially a multiplicity of cross-linkers on the fibronectin, and since a nitrene may react with its own protein rather than cross-linking to another protein, these values need not be identical. For example, in the samples in line 7, 10–18% of the cross-links were between fibronectin and gelatin-Sepharose (see Table I, line 8) while the others must be within or between fibronectin molecules which are removed by the SDS in line 8.

Therefore, in this favorable situation, more than one-third of the known fibronectin-gelatin contacts become converted into covalent bonds and, after reduction, 10–20% of the distal parts of the cross-linker remain covalently attached to the target (gelatin beads).

Cross-linking Gelatin-XL to Fibronectin in Solution—To determine the ratio of specific to nonspecific cross-linking, we studied the cross-linking of gelatin-XL to fibronectin in the presence of carrier proteins which do not interact with either. Gelatin-XL and fibronectin were mixed in solution with or without variable amounts of other proteins, photolyzed, and reduced. Controls were kept in the dark and unreduced. The proteins were separated by SDS-PAGE, and radioactivity determined by autoradiography.

The results of an experiment in which gelatin-XL was mixed with fibronectin alone are shown in Fig. 3. The unphotolyzed and unreduced samples show the characteristic gelatin pattern with $\alpha_1$ and $\alpha_2$ chains, and the $\beta$ dimer (tracks 1 and 3). When photolyzed, the gelatin bands greatly diminish and the radioactivity at the top of the gel increases, indicating the formation of cross-linked complexes (tracks 2 and 4). On reduction of unphotolyzed samples, nearly all the radioactivity is lost (tracks 5 and 7). On reduction of photolyzed samples, most of the radioactivity moves down into the gelatin and fibronectin bands (tracks 6 and 8). This is the behavior one would expect according to Fig. 2, when gelatin corresponds to P1, and fibronectin to P2.

To test for nonspecific cross-linking (Fig. 2, steps 5c and 6c), we repeated this experiment with excess carrier proteins (Fig. 4). In the presence of a 5–50-fold mass excess of human IgG and chicken ovalbumin, the nonreduced samples (data not shown) appeared very similar to those in Fig. 3 (tracks 1–4). The reduced samples (Fig. 4A) were also similar to those in the experiment without carrier, although self-labeling of gelatin occurred at a lower level. Most important, even in the presence of a 50-fold mass excess (150- and 500-fold molar excess for IgG and ovalbumin, respectively), the specific labeling of fibronectin was only slightly decreased, and the nonspecific labeling of carrier proteins was barely detectable.

This experiment was also carried out using an NP40 extract of NIL8 cells as carrier protein (Fig. 4B), and showing the dependence of the transfer of label on the fibronectin concentration. In the presence of the complex mixture of proteins and NP40 detergent, some label appeared on top of the gel, but no other proteins appeared as labeled bands on the gel. The label at the top of the gel could be nonspecific trapping during electrophoresis or could represent reaction of cross-linker with large molecules such as proteoglycans, which are known to interact both with collagen and with fibronectin.

These experiments indicate that large excesses or complex mixtures of carrier proteins do not pick up radioactivity from the cross-linker-modified gelatin and that cross-linking and transfer of label to fibronectin occurs specifically and efficiently.

Additional experiments of this general type did however reveal potential difficulties with nonspecific labeling. Fig. 5A shows a nonreduced gel of an experiment in which gelatin-XL was mixed with a very large excess of human serum. In tracks 1 and 2 one can see that most of the radiolabel has been transferred from the gelatin to the serum albumin, with or without photolysis. Nearly all of this label was removed from the albumin in the unphotolyzed sample by reduction (not shown), suggesting that it had become associated with the free sulphydryl on the albumin by disulfide interchange. When ethyl mercury phosphate, which reacts very rapidly with sulphydryl groups to form a tight complex, was added, transfer of radiolabel to albumin was prevented (tracks 3 and 4), which supports this hypothesis. The experiment shown in Fig. 4B
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**Fig. 2. Cross-linking scheme.** I, The succinimide ester moiety on the cross-linking reagent reacts with amino groups on proteins to form an amide linkage. $^3$S denotes the radiolabeled sulfur. $^3$S label allows determination of the number of cross-linkers per molecule of protein. 2, the labeled protein ($P_1$) is reconstituted with other proteins, cells, etc. In general, there will be interacting proteins ($P_2$) and noninteracting proteins ($P_3$). 3, at any stage before photolysis, reduction cleaves the disulfide link to remove the radiolabeled sulfur. No proteins should remain labeled. 4, photolysis converts the azide group to a highly reactive nitrene. 5, the nitrene can react at a second site to form a covalent bond. In general, this may take the form of self-reactions (a), specific cross-linking (b), nonspecific cross-linking (c), or abortive reaction with solvent (d). If samples are analyzed at this point without reduction, radiolabel will be found both in the starting protein $P_1$, and in various higher molecular weight complexes. 6, reduction cleaves the disulfide link so that the radiolabel is associated with the second reaction site. The reaction products in 6 lead to self-labeling (a), specific labeling of proteins which interact with $P_1$ (b), and nonspecific labeling of proteins which are nearby but do not directly interact with $P_1$ (c). Our results show that nonspecific labeling (c) is very low whereas specific labeling (b) is readily detected.

was also conducted in the presence of ethyl mercury phosphate and, although a detergent extract of cells would be expected to contain many free sulphydryls, nonspecific transfer of label was very low.

Fig. 5B shows the cross-linking between gelatin-XL and fibronectin in the presence of a high concentration of purified BSA and ovalbumin. Even with ethyl mercury phosphate, the BSA, though not the ovalbumin, did pick up a small quantity of radioactivity from the gelatin-XL. Table II shows the quantitation of these results.

In the sample with a 40-fold mass excess of BSA and ovalbumin, there is approximately 4 times the radioactivity associated with the fibronectin band as with BSA, and over 20 times as much as with ovalbumin. If these values are normalized for the large mass excess of carrier protein, we calculate that the cross-linker exhibits a preference for fibronectin over carrier which is approximately 160 for BSA and 900 for ovalbumin. In the experiment where IgG and ovalbumin were present in excess, the cross-linker showed a 400-fold preference for fibronectin over IgG, and an 800-fold preference over ovalbumin. These values are likely to underestimate the specificity of the cross-linker for fibronectin, since gel bands with such a high concentration of protein will tend to concentrate background radioactivity. The background appearing at

| Table I
| Cross-linking of fibronectin XL to gelatin-Sepharose |
| Treatment | Radioactivity bound to gelatin-Sepharose | Photolysis-dependent reaction |
| Light | DTT | SDS | Experiment 1 | Experiment 2 | Experiment 1 | Experiment 2 |
|-------|-----|-----|-------------|-------------|-------------|-------------|
| 1     | -   | -   | -           | 81          | 69          |             |
| 2     | -   | -   | +           | 10          | 13          |             |
| 3     | -   | +   | -           | 3           | 5           |             |
| 4     | -   | +   | -           | 8           | 6           |             |
| 5     | +   | -   | -           | 100         | 100         |             |
| 6     | +   | +   | -           | 46          | 54          | 36          | 41          |
| 7     | +   | -   | +           | 43          | 29          | 40          | 24          |
| 8     | +   | +   | +           | 26          | 15          | 18          | 10          |

Fig. 3. Cross-linking in solution. Gelatin-XL in solution, alone (tracks 1, 2, 5 and 6) or with fibronectin (tracks 3, 4, 7 and 8) were photolyzed (+) or not (-) as indicated above each track. Samples in tracks 1-4 were kept unreduced, while those in tracks 5-8 were reduced with dithiothreitol. Tracks 1 and 3 show the initially labeled gelatin. Tracks 2 and 4 show the formation of large cross-linked complexes upon photolysis. When reduced, the radiolabel is removed from unphotolyzed samples as shown by tracks 5 and 7. Track 8 shows that radiolabel is transferred to fibronectin after photolysis and reduction.
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Single new protein with the molecular weight of fibronectin becomes labeled. Therefore, in this biological system, there was specific cross-linking of gelatin to fibronectin in the absence of any detectable nonspecific cross-linking. An inhibitor of disulfide interchange during the binding and cross-linking states was not necessary, presumably because few sulfhydryls

the leading edge of the band will be counted as part of the band. The quantity of label which is covalently linked to the carrier proteins is probably smaller than the values determined by scanning the gel.

Cross-linking Gelatin-XL to Fibronectin on Cells—To determine if this method is applicable to proteins on cell surfaces, we attempted to cross-link gelatin to fibronectin on NIL8 fibroblasts. These cells were incubated with gelatin-XL in the dark, washed to remove unbound gelatin, and the suspension photolyzed and reduced. The cells were lysed in 2% SDS containing ethylmercury phosphate to block disulfide exchange and run on SDS-PAGE.

Fig. 6 shows that after photolysis and reduction, only a single new protein with the molecular weight of fibronectin becomes labeled. Therefore, in this biological system, there was specific cross-linking of gelatin to fibronectin in the absence of any detectable nonspecific cross-linking. An inhibitor of disulfide interchange during the binding and cross-linking states was not necessary, presumably because few sulfhydryls

Fig. 4. Specificity of cross-linking. A, gelatin-XL in solution, alone (tracks 1 and 2) or with 10 μg of fibronectin (tracks 3-10) was also incubated with various amounts of IgG and ovalbumin. The samples in tracks 5 and 6 have 50 μg of each protein, tracks 7 and 8 have 150 μg, and tracks 9 and 10 have 500 μg. Samples were photolyzed (+) or not (−) as indicated above each track. All samples were reduced with dithiothreitol and run on an 8% polyacrylamide gel. Arrows mark the positions of fibronectin (FN), gelatin α1α2, and β chains, IgG heavy (H) and light (L) chains, and ovalbumin (O). B, gelatin-XL was incubated with 30 μg (tracks 1, 2, 5 and 6) or 100 μg (tracks 3, 4, 7 and 8) of fibronectin. Samples in tracks 5-8 also received approximately 1 mg NIL cell NP40 extract. Photolysis and the location of protein bands are indicated as in A. All samples were reduced and run on a 7% polyacrylamide gel.

Fig. 5. Disulfide interchange. A, gelatin-XL plus human serum was incubated without (tracks 1 and 2) or with ethylmercury phosphate (tracks 3 and 4). Photolysis is indicated above each track by a plus or minus. All samples were run unreduced in an 8% polyacrylamide gel. Arrows indicate positions of gelatin (α1α2) and serum albumin (HSA). B, gelatin-XL with 25 μg of fibronectin was incubated with varying amounts of BSA and ovalbumin in the presence of ethyl mercury phosphate. Samples in tracks 1 and 2 received 100 μg each, tracks 3 and 4 received 300 μg each, and tracks 5 and 6 received 1000 μg each. All samples were reduced and run on an 8% polyacrylamide gel. Photolysis and the location of protein bands are indicated as in Fig. 4.
TABLE II
Specific and nonspecific transfer of radioactivity from gelatin-XL

Quantitation was performed by scanning densitometry of the autoradiographs shown in Fig. 4 and 5. Values are given as percentages of the total density in the defined bands in each track, minus background. In sample lines 2, 3, and 4 there is a 4-, 12-, and 40-fold mass excess of each carrier protein, over fibronectin, respectively. In line 5, from a separate experiment, there is a 50-fold excess. FN, fibronectin.

| Sample | Fibronectin | BSA heavy | IgG light | Ovalbumin |
|--------|-------------|-----------|-----------|-----------|
| 1 25 μg of FN alone | 88 | 7 | 0 |
| 2 FN + 100 μg of BSA and ovalbumin | 70 | 14 | 2 |
| 3 FN + 300 μg of BSA and ovalbumin | 69 | 18 | 3 |
| 4 FN + 1000 μg of BSA and ovalbumin | 84 | 8 | 2 |
| 5 FN + 500 μg of IgG and ovalbumin | 88 | 7 | 0 |

Fig. 6. **Cross-linking at the cell surface.** Gelatin-XL was allowed to bind to NIL8 cells and the cell layers dissolved in SDS. Samples in *tracks* 1 and 2 were nonreduced, while *tracks* 3 and 4 were reduced with dithiothreitol and alkylated with iodoacetic acid, and all were run on a 7% polyacrylamide gel. Photolysis and the reduction of protein bands are indicated as in previous figures. After photolysis and reduction (*track* 4), fibronectin is specifically labeled, whereas in the absence of photolysis there are no labeled bands after reduction (*track* 3).

are present outside cells. Therefore, the cross-linking reagent can be used to cross-link cell surface molecules under physiological conditions.

**DISCUSSION**

Chemical cross-linking has been of some use in elucidating proximity relationships in ribosomes, chromatin, and oligomeric proteins, but has generally been of more limited value for studying protein-protein interactions in complex systems (1). This is largely for two reasons. First, when many proteins are present and the number of interactions is large, the resulting gel pattern of cross-linked complexes is generally too complicated to extract much information. This is aggravated by the high molecular weights of most cross-linked complexes. Second, even when additional techniques, such as immuno-precipitation, are employed for selecting specific complexes and simplifying the patterns, the total background often remains too high to permit resolution of minor species.

In this paper, we report the development of a radioactive, cleavable, photoactivatable cross-linking reagent. This reagent may be used to make essentially any protein into a photoaffinity probe which, after photolysis and reduction, will donate a radioactive moiety to adjacent molecules. These can then be analyzed by SDS-PAGE or other standard techniques.

This method offers several advantages over conventional cross-linking reagents. First, since the phenylazide is inert towards proteins until it is irradiated, while the succinimide ester reacts with amino groups immediately, a purified protein can be derivatized with the reagent, repurified, and then reconstituted into the biological system. Cross-linking will not occur until it is photolyzed. This assures that all cross-linked complexes involve the protein under study, and simplifies the pattern of cross-linked complexes enormously.

Second, the nitro group on the phenyl ring enables visible and near-UV radiation to activate the azide moiety, thereby reducing the possibility of radiation damage to proteins during photolysis. The nitro group may also contribute to the very high yield of cross-linked product by stabilizing the nitrene generated on photolysis and so increasing its lifetime.

Third, the radiolabel provides a convenient means of assaying the number of cross-linkers in the derivatized protein, and for following it in subsequent manipulations. This makes it possible to control the number of cross-linkers per protein. Since the reagent can be synthesized with very high specific activity, only one or a few cross-linkers per protein will usually be necessary, so that the protein will be altered as little as possible. This contrasts with nonradioactive reagents which are sometimes used at mole ratios of 100:1 (3).

Fourth, the radiolabel provides a means for studying systems where radioactivity cannot be introduced by other means.

Fifth, the radiolabel, combined with cleavability, provides a means for identifying neighboring proteins with very high signal-to-noise ratios. Only those proteins sufficiently close to the initially labeled protein become labeled after photolysis and reduction.

Sixth, the nitrene generated by photolysis of the azide is sufficiently reactive to be able to form covalent links to essentially any biological molecule (1, 21, 22). The reaction does not require any particular functional groups to be present in the neighboring molecules, so that lipids, carbohydrates, or nucleic acids as well as proteins can be cross-linked.

Finally, since the cross-linker labels the distal partner of the complex, the products can be analyzed after reduction, thereby avoiding the problems associated with analysis of high molecular weight complexes.

We have presented evidence that this reagent exhibits the proper chemical behavior under biological conditions. By measuring incorporation of radioactivity into the protein peak on a gel filtration column, we found that the cross-linker can be covalently coupled to proteins with yields in the range of 15-50%. When attached in the dark, the radiolabel is removed by dithiothreitol, indicating that the radioactivity is on the distal sulfur of the disulfide bond. On photolysis, the azide is converted to a highly reactive nitrene which can react to form a covalent bond at a second site, either on the initial protein or on a neighboring one; we found that on the order of 25-40%
of the initial radioactivity became dithiothreitol-resistant after photolysis (Table I).

Our results also show that when a cross-linker-derivatized protein is allowed to bind a second protein and then photolyzed, covalent cross-links are formed between the two, as judged by resistance to boiling in SDS. After treatment with dithiothreitol, the cross-link is cleaved and the second protein becomes radiolabeled. In one experimental system we found that on the order of 10–20% of the initial radiolabel was transferred to the second protein. When a noninteracting carrier protein was present, or when the reaction was carried out at the cell surface, very little nonspecific cross-linking was observed, even when carrier protein was in large excess. In the worst case, using as carrier protein BSA, which is known to bind hydrophobic molecules like the cross-linker, we found that the ratio of specific to nonspecific cross-linking was approximately 160:1. In more favorable cases the ratio was 400:1 and 900:1.

Several potential difficulties associated with this method may be worth considering. The first and probably most severe problem is disulfide exchange. When proteins containing exposed free sulfhydryl groups are present, exchange probably will occur to some extent. The use of blocking reagents may not always be feasible, for example, when studying proteins on live cells where blocking sulfhydryl groups could have many uncontrolled effects. Fortunately, the concentration of free sulfhydryl groups outside the cell is low. In the experiment where gelatin-XL was cross-linked to fibronectin at cell surfaces, disulfide exchange was too low to be observed (Fig. 6). When the level of exchange is higher, inclusion of unphotolyzed and nonreduced samples in the experiments should provide adequate controls. However, disulfide exchange may preclude using this reagent in the cytoplasm where the concentration of free sulfhydryl groups is much higher.

The second difficulty, if it can be called a difficulty, stems directly from the primary advantage of chemical cross-linking, which is its generality. Cross-linking provides information concerning protein-protein contacts, but has no bearing on the functional significance of these contacts. If two proteins are found in the same region of the membrane, cross-links could be formed between them even though they do not directly interact. This sort of information may be of interest, but in general, experiments must be designed to test the functional significance of cross-links, and results must be interpreted with this in mind.

The third difficulty is that, as in any study where a probe is attached to a protein, the protein may be altered by its modification. This can be minimized by ensuring that only one cross-linker is attached per protein, but it remains a problem. The cross-linker moiety is strongly hydrophobic, and could conceivably induce spurious interactions, for example with the lipid bilayer or with hydrophobic regions of proteins. In all studies of this sort, the modified protein must be tested to be sure its properties have not been altered, and experiments should include adequate controls, such as adding excess unlabeled protein to compete binding of modified protein.

In conclusion, we have shown that this cross-linker is a sensitive and specific reagent for studying protein-protein interactions. We have shown that in model systems it exhibits the proper chemical behavior, can identify protein-protein contacts with a high signal to noise ratio, and can successfully identify proteins on the surfaces of living cells in culture. We have also identified several potential difficulties, and have attempted to determine to what extent they limit this method.

We are currently using this reagent to study the interactions of fibronectin on fibroblast surfaces, in phagocytosis by macrophages, and in hemostasis. This method should also be useful for identifying virus receptors, antigen and hormone receptors, actin or tubulin binding proteins, and for many other problems of this sort.

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