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MEMBRANE-IMPERMEANT, CLEAVABLE CROSS-LINKERS

NEW PROBES OF NEAREST NEIGHBOR RELATIONSHIPS AT ONE FACE OF A MEMBRANE

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We have developed a new type of chemical probe for the determination of nearest neighbor relationships of membrane components at one face of a membrane: membrane-impermeant, cleavable cross-linkers. These bidentate compounds form covalent adducts with primary amino groups and can therefore be used to probe protein-protein and, in principle, protein-aminolipid and aminolipid-aminolipid interactions. An internal disulfide bond in each of the cross-linkers allows the cleavage of the cross-linking bridge to facilitate the analysis of the components of the cross-linked complex. What distinguishes these reagents from other cleavable cross-linkers (reviewed in reference 1) is that these probes do not permeate biological membranes, so that one can determine not only what interactions occur in a membrane, but also at which face of a membrane they occur.

RESULTS AND DISCUSSION

We have synthesized prototypes of two chemical classes of membrane-impermeant, cleavable cross-linkers (Fig. 1). Diisethionyl-3,3'-dithiobispropionimidate (DIDIT) is a bis(alkyl imidate) which forms amidines from primary amino groups (2) (Fig. 1, Eq. I). 3,3'-Dithiobis(sulfosuccinimidylpropionate) (DTSSP) is a bifunctional active ester which forms amide linkages from primary amines[1] (Fig. 1, Eq. II).

The chemical properties of these compounds are complementary. Alkyl imidates, such as DIDIT, have the distinct advantage that the product amidines (Fig. 1, Eq. I) are charged at neutral pH like the primary amines from which they were derived. Even extensive amidination does not markedly change the physical properties of many proteins (3). However, the pH maximum for the reaction of alkyl imidates with primary amines is far above physiological, e.g., pH 9–10.5 for methyl acetimidate with α- or ϵ-amino groups (4). Even when the reaction is carried out at high pH, hydrolysis can effectively compete with aminolysis, reducing the yield of the cross-linking reaction. Niehaus and Wold (5) showed that when erythrocyte membranes are reacted with dimethyladipimidate at pH 9.6, only 20% of the modified lysines were involved in cross-links; the remainder were modified with reagent which had hydrolyzed at its second reactive functionality. Further, when the reaction is carried out below pH 8, a side reaction becomes significant in which *N,N'*-disubstituted amidines (which constitute non-cleavable cross-links) are formed (6).

The benefits and liabilities of active esters such as DTSSP are essentially the converse of those for alkyl imidates. The aminoacyl adduct formed (Fig. 1, Eq. II) is not charged at neutral pH, since on reaction, the primary amino group becomes an amide nitrogen. Therefore, one must be cognizant of potential structural changes on reaction due to a change in charge. However, the reaction can be carried out at neutral pH; by analogy with the closely related *N*-hydroxysuccinimide esters (7), the rates of hydrolysis are very slow, so the chemical yield of cross-linked products can be relatively high.

We have tested both DIDIT and DTSSP using a series of procedures designed to show that the probes are cross-linkers, that they are cleavable, and that they are membrane-impermeant (2). Rabbit muscle aldolase, a tetrameric protein having four identical 40,000 dalton subunits, was treated in solution with a series of concentrations of each reagent, and the products were subjected to electrophoresis in sodium dodecyl sulfate-polyacrylamide gels. When gels were run under non-reducing conditions, bands were observed corresponding to dimers, trimers, tetramers and, at higher reagent concentrations, traces of

[1]The chemistry of *N*-hydroxysulfosuccinimide and of the active esters formed with this compound is discussed elsewhere (Staros, J. V., in preparation).

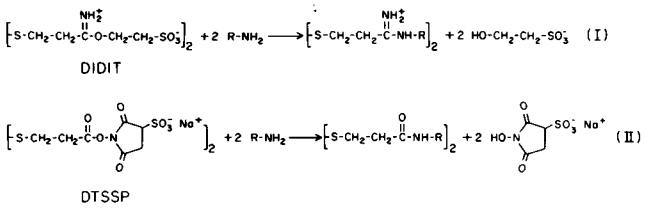

FIGURE 1 Membane-impermeant, cleavable cross-linkers and their reactions with primary amines.

higher oligomers as well as monomers of aldolase subunits. The same reaction mixtures subjected to electrophoresis under reducing conditions yield predominantly the band corresponding to aldolase monomers. These studies indicate that the reagents function as cleavable cross-linkers.

Isolated human erythrocyte membranes were reacted with either probe, and the products of reaction were analyzed by two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis in which the first dimension is run under non-reducing conditions and the second dimension is run under reducing conditions to cleave the cross-link (8). While the gel patterns obtained with the two cross-linkers are not identical, both yield evidence of extensive cross-linking of cytoplasmic face membrane proteins, cytoskeletal proteins, and residual hemoglobin. However, when membranes isolated from intact erythrocytes which had been reacted with either probe were subjected to the same two-dimensional electrophoresis as above, the electropherograms contained no evidence of the cross-linking of cytoplasmic face membrane proteins, cytoskeletal proteins, or hemoglobin. The major product of cross-linking is a dimer of band 3 (anion channel) subunits cross-linked at the extracytoplasmic membrane face (2).

The ability to cross-link membrane components at one face of a membrane makes accessible three-dimensional information concerning the interactions among those components, i.e., one can pose not only the question of which components are adjacent to which others, but also the question of at which face such interactions occur. Membrane-impermeant, cleavable cross-linkers should prove useful in many studies of membrane topology, including investigations of subunit-subunit contacts in oligomeric membrane proteins, proximities of secondary structural domains within membrane proteins, and interactions of polypeptide hormones with their membrane receptors.

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