

METHOD OF CROSSLINKING AMINO ACID CONTAINING POLYMERS USING PHOTOACTIVATABLE CHEMICAL CROSSLINKERS

This is a continuation of co-pending application Ser. No. 07/159,603, filed on Feb. 24, 1988.

FIELD OF THE INVENTION

This invention relates to methods for molecularly crosslinking amino acid containing polymers by photoactivating chemical crosslinking reagents which have been combined with the polymers. More particularly, the invention relates to methods for molecularly crosslinking collagen by photoactivating heterobifunctional crosslinking reagents which have been combined with collagen. Upon photoactivation, reactive groups on these bifunctional reagents crosslink the collagen by forming bridges between amino acid side chains on the collagen molecule.

BACKGROUND OF THE INVENTION

Chemical crosslinkers have been used to study the molecular organization of cell membranes and to understand the way in which various molecules interact with one another at the inner or outer surface of the membrane (Peters, K., Richards, F. M., *Ann. Rev. Biochem.* 46:523-51, 1977). Protein structural studies utilizing chemical crosslinking began during the 1950's with the work of Zahn (*Angew. Chem.* 67:561-572, 1955; *Makromol. Chem.* 18:201-216, 1955; *Makromol. Chem.* 72:126-152, 1958) and continued in the 1960's, primarily with the work of Wold and his colleagues (*J. Biol. Chem.* 236:106-111, 1961). In addition, crosslinkers have been used to artificially crosslink and stabilize tissue (Nimni, M., *Biorheology*, 17:5182 1980).

Crosslinking techniques for the membrane system studies discussed above have made use of bifunctional reagents, which are classified as either homo- or heterobifunctional. Homobifunctional reagents have two identical reactive sites. Heterobifunctional reagents carry two dissimilar binding sites, one photosensitive and one conventional site. In general, both types of bifunctional reagents act to form chemical crosslinks by introducing bridges between amino acid chains.

The utility of the homobifunctional reagents as crosslinkers in membrane studies has been limited due to several potential inherent problems including random collisional crosslinks, long reaction time, difficulty in controlling reactions and nonselective crosslinking. Random collision-dependent crosslinks can occur at a significant frequency, since molecules nonspecifically crosslink during random collisions in fluid membranes. Such indiscriminate formation of crosslinks can result in a high multiplicity of crosslinked products which are difficult to analyze. It is possible therefore, that low yield crosslinked products would go undetected. These random collisional crosslinks were avoided in some membrane systems with the use of rapidly crosslinking photosensitive agents. (Ji, T. H., *Biochimica et Biophysica Acta*, 559: 39-69 1979).

In contrast, crosslinking with photosensitive heterobifunctional reagents, can be easily, rapidly and sequentially controlled. Crosslinking with heterobifunctional reagents is accomplished by binding the conventional site on the reagent to one amino group via an amide bond, leaving the second photoactivatable site

unbound. Upon photoactivation by the use of ultraviolet or visible irradiation, the photoactivatable site is converted to a species of very high chemical reactivity, which then forms a covalent linkage with another amino acid side chain.

The absorption of ultraviolet or visible radiation by the bifunctional reagent can give rise to two general classes of species produced by cleavage of chemical bonds. Fragmentation can be either at a single bond, resulting in the formation of two free radicals, or at a double bond to carbon or nitrogen. Two types of photosensitive groups are known that result from cleavage at a double bond to carbon or nitrogen: an azide derivative and a diazo derivative. Nitrenes are generated from azides, and carbenes are generated upon photolysis of diazo derivatives. Both nitrenes and carbenes are compounds of very high chemical reactivity.

A common method used for photoactivation of heterobifunctional compounds is irradiation with a short wave ultraviolet lamp, for example, mineral light USV-11. The half time of photolysis with this lamp varies depending on the reagents and is in the order of 10 to 50 seconds. An alternative method, which has several advantages, is flash photolysis for an extremely short period, normally on the order of milli seconds.

Collagen is the single most abundant animal protein. It is the main structural component of mammalian tissues and accounts for about 30% of all mammalian proteins (Nimni, M., *Biorheology*, 17:51-82, 1980). The molecular structure of collagen consists of three intertwining helical polypeptide chains about 1,050 residues long, wound around each other to form a triple helix.

There is a great amount of uniformity in the amino acid composition of collagen. Glycine forms about 33 percent and proline and hydroxyproline form about 25 percent of the total amount of residues in the polypeptide chains. Proline and hydroxyproline contribute to the rigidity of the molecule in that the beta C is linked to the peptide nitrogen by the side chain, forming a five membered ring thus allowing relatively little freedom of rotation. It is this locking effect by proline and hydroxyproline residues, and the hydrogen bond formation by the hydroxyl group of hydroxyproline, which gives collagen its great stability. The other amino acid residues in the structure include 10 percent alanine and 20 percent polar side chains of arginine, lysine, asparagine and glycine. These do not play a particularly important role in the triple helix but nevertheless are important in the intermolecular linkages which lead to fiber formation.

Crosslinking of the collagen molecules occurs extracellularly and leads to formation of the collagen fiber. This characteristic fiber organization is responsible for the functional integrity of tissues such as bone, cartilage, skin and tendon, and for the structural integrity of blood vessels and most organs.

Both intra- and intermolecular crosslinks in collagens are derived from lysine and hydroxylysine residues. Intramolecular crosslinks are formed when specific lysine and hydroxylysine residues in collagen are oxidatively deaminated to peptide bound aldehydes. Copper, a co-factor with the enzyme lysyl oxidase, causes this modification to take place. The actual formation of the crosslinks takes place via aldol condensation, a spontaneous non-enzymatic reaction where the lysines which are located near the end-terminal region are converted to aldehydes. Intermolecular crosslinks are formed between peptide bound aldehydes and unmodified amino