How to cross-link proteins

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Background

Protein-protein interactions comprise the underlying molecular mechanism of a multitude of complex biological processes. Interaction between intracellular proteins can be transient, wherein two or more proteins associate in the catalysis of a step in a biosynthetic or signal-transduction pathway; alternatively, stable multiprotein complexes are required in the performance of several biological functions. Molecular chaperone complexes represent a very good example of multi-protein assemblies where the cooperative action of several components is required for folding/unfolding and transmembrane trafficking of proteins. Signal transduction pathways are illustrative of highly complex protein interaction networks displaying transient and semi-stable association of a series of proteins. Chemical crosslinking offers a direct method of identifying both transient and stable interactions. This technique involves the formation of covalent bonds between two proteins by using bifunctional reagents containing reactive end groups that react with functional groups—such as primary amines and sulfhydryls—of amino acid residues. In the cell, a single protein may often engage in transient interaction with a variety of other partners in a given pathway. Where purified proteins are available, chemical crosslinking is the ideal strategy for an unambiguous demonstration of protein-protein interactions, *in vitro*. If two proteins physically interact with each other, they can be covalently cross-linked. The formation of crosslinks between two distinct proteins is a direct and convincing evidence of their close proximity. In addition to information on the identity of the interacting proteins, crosslinking experiments can reveal the regions of contact between them.

Crosslinking agents: Crosslinkers (CL) are either homo- or hetero-bifunctional reagents with identical or non-identical reactive groups, respectively, permitting the establishment of inter- as well as intra-molecular crosslinkages. Inter-subunit crosslinks have been used

for determination of the quaternary structure and arrangement of subunits within homo-oligomeric proteins and intra-subunit crosslinks for maintenance of stable tertiary structure. Ligand-induced conformational changes in proteins can be analyzed by a comparison of the rate/extent of crosslinking in the ligand-bound *versus* the unliganded states. In studies of hetero-oligomeric enzymes or multiprotein complexes—containing several different polypeptides—a crosslinking offers a reliable tool for unraveling spatial relationships of the components. A variety of crosslinkers are commercially available from major suppliers such as Pierce, Molecular probes, and Sigma. For an insightful treatment of the properties and underlying chemistry of crosslinkers, and methods of introducing reactive groups into proteins, the all-time classic by Means and Feeney (1971) should be consulted.

Homo-bifunctional reagents, specifically reacting with primary amine groups (*i.e.*, ε-amino groups of lysine residues) have been used extensively as they are soluble in aqueous solvents and can form stable inter- and intra-subunit covalent bonds. Glutaraldehyde, a popular reagent, has been used in a variety of applications where maintenance of structural rigidity of protein is important. Homo-bifunctional imidoesters, with varying lengths of the spacer arm between their reactive end groups, are particularly useful in determination of the distances between linked residues on surfaces of neighbouring subunits in oligomeric proteins. Some examples of imidoesters are dimethyl adipimidate (DMA), dimethyl suberimidate (DMS) and dimethyl pimelimidate (DMP) with spacer arms of 8.6Å, 11Å and 9.2Å, respectively. DMA and DMS have been used to verify the quaternary structures of many oligomeric enzymes.

For identification of unknown interacting partners of a given target protein, reversible homo-bifunctional crosslinkers such as N-hydroxysuccinimide (NHS) esters are advantageous in that the interacting proteins can be recovered and identified. Examples of reversible NHS-esters are DSP, dithiobis(succinimidylpropionate), which is water insoluble and the soluble derivative dithiobis(sulfosuccinimidylpropionate), DTSSP. These reagents can be cleaved by treatment with thiols, such as β–mercaptoethanol or dithiothreitol. A crude cellular extract is treated with the crosslinking reagent and immunoprecipitation with antibodies specific for the target protein is used to recover the assorted complexes containing it. The immunoprecipitate is then treated with

a reducing agent thereby separating the target from its partner protein(s). This can be done simply by dissolving the immunoprecipitate in the denaturing sample buffer (Laemmli 1970), prior to resolution by SDS-PAGE. The stained, polypeptide bands, corresponding to the partner proteins, can be excised and identified by MALDI-TOF mass spectrometry, following in-gel trypsin hydrolysis.

Protein-protein interaction can also be analyzed by employing hetero-bifunctional reagents that can form stable thioester bonds between two interacting proteins. For instance, crosslinkers with one amine-reactive end and a sulfhydryl-reactive moiety at the other end are selected in situations where the catalytic site of one of the protein contains an amine. To avoid damaging the active site, it is preferable to target other side chains for modification. Hetero-bifunctional crosslinkers with a NHS ester at one end and an SH-reactive groups—maleimides or pyridyl disulfides—can be used. Another approach is to modify the lysine residues of one protein to thiols and the second protein is modified by addition of maleimide groups followed by formation of stable thioester bonds between the proteins. If one of the proteins has native thiols, these groups can be reacted directly with maleimide attached to the other protein. Alternatively, hetero-bifunctional reagents containing a photoreactive group, such as Bis[2-(4-azidosalicylamido)ethyl)] disulfide, BASED, can also be used. In the next step, the reaction between the two proteins is performed giving rise to stable thioester bonds. With all hetero-bifunctional reagents the crosslinking experiment is conducted in two steps. One protein is modified first with one reactive group of the hetero-bifunctional reagent; the remaining free reagent is removed. In the next step the modified protein is mixed with the second protein, which is then allowed to react with modifier group at the other end of the reagent. Some examples of such reagents, among others, are SPDP [succinimidyl 3-(2-pyridyldithio)propionate] and a related compound, SMCC [succinimidyl *trans*-4-(maleimidylmethyl)cyclohexane-1-carboxylate], supplied by Pierce; several others are listed in the Fluka catalogue.

Procedures

Before starting crosslinking experiments, it is important to select the requisite crosslinker for optimal reaction with the proteins. Features such as solubility, membrane permeability, spacer arm length and reversibility of cross-links should be taken into

consideration. Moreover, the experimental procedure should be such that it can be conducted under mild conditions of pH and temperature to preserve the native structure of the proteins. Homo-bifunctional reagents such as DMS, DMA, and glutaraldehyde work very well with *Neurospora* proteins.

Glutaraldehyde: Treatment with crosslinkers should be conducted in buffers free from amines. Phosphate buffers at pH 7.5 to 8.0 and HEPES buffers are suitable whereas, Tris-HCl should be avoided. For glutaraldehyde treatment, reaction mixtures with 50 to 100 µg of interacting proteins in 20 mM HEPES buffer (pH 7.5) in a total volume of 100 µl are treated with 5 µl of 2.3% freshly prepared solution of glutaraldehyde for 2 to 5 minutes at, 37^{o}C. The reaction is terminated by addition of 10 µl of 1 M Tris-HCl, pH 8.0. Cross-linked proteins are solubilized by addition of an equal volume of Laemmli sample buffer to which a few µl of 0.1% bromophenol blue is added and electrophoresis is conducted in 5% to 20% SDS-polyacrylamide gels.

Dimethyl adipimidate (DMA) and Dimethyl suberimidate (DMS): Crosslinking reactions with DMA and DMS are performed in 20 mM buffer (pH 7.5). The crosslinkers are dissolved in the buffer to a concentration of 6 mg/ml and pH is adjusted to 8.5 by addition of NaOH. A solution of 1mg/ml of protein containing a concentration of the diimidoester at 1 or 2 mg/ml is allowed to crosslink for 3 hours at room temperature. The reaction is terminated by addition of the denaturing sample buffer, bromophenol blue and the mixture is resolved by SDS-PAGE.

Stained gels of oligomeric proteins, for instance tetrameric proteins, on treatment with the above homo-bifunctional reagents will show crosslinked dimers, trimers and tetramers, where inter-subunit crosslinking has occurred, as well as crosslinked monomers with intra-subunit crosslinks, along with the unmodified monomers. Computer-assisted densitometric analysis can be used to estimate the relative quantities of monomeric, dimeric, trimeric and tetrameric species, resolved by SDS-PAGE. If the arrangement of subunits in the native tetramer is symmetrical, the contacts between all of the individual protomers in the quaternary structure should be identical. On statistical, theoretical grounds, therefore, the frequency of single crosslinks per tetramer will be the

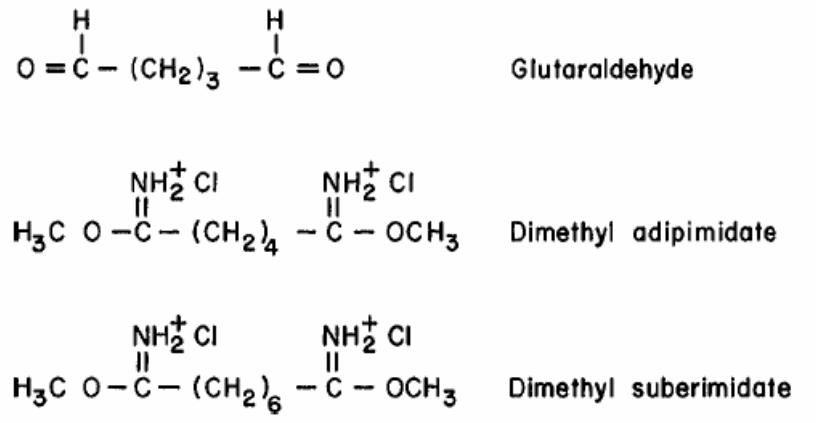
higher than that for two crosslinks and that for 3 and 4/tetramer will be correspondingly lower (uncross-linked monomer > dimer > trimer > tetramer). If the arrangement of protomers in the tetramer is asymmetrical, the distribution of cross-linked species will not conform to this pattern. A preponderance of cross-linked dimeric species, relative to monomers and crosslinked trimers and tetramers is indicative of a "dimer-of-dimer" structural arrangement.

Reversible, thio-cleavable bifunctional reagents: The advantage of using reversible reagents is the ability to liberate the participating proteins from the cross-linked product. As a result, proteins interacting with a given protein (X) can be identified. Crosslinking reactions are conducted by mixing crude cellular extracts or partially purified samples, with protein X. Crosslinking of *Neurospora* proteins, in a crude extract, works well with DSP. The reagent is dissolved in DMSO at 25 mM. The reaction buffer for cross-linking is 20 mM sodium phosphate, 0.15 M NaCl, pH 7.5 (PBS), or HEPES may be used instead. To the protein solution (0.25 to 1 mg/ml containing protein X and the crude extract) is added DSP stock solution to a final concentration of 2 mM. After incubation for 2 hours on ice, 1 M Tris-HCl (pH 7.5) is added to a concentration of 20 mM to stop the reaction. The mixture is incubated for 15 min. The complexes are recovered by overnight treatment with an antibody-matrix (immobilized anti-X IgG) and the immunoprecipitate is collected by centrifugation. The precipitate is washed with PBS to remove the unbound material and Dithiothreitol (50 mM final concentration) is added and following incubation at 37^{o}C for 30 min the mixture is resolved by SDS-PAGE. The gel is stained with Coomassie blue and the polypeptide bands of interacting proteins are excised, subjected to in-gel trypsin hydrolysis and the sample prepared for MALDI-TOF mass spectrometric analysis for identification of the protein. This procedure has been used for analysis of proteins, interacting with Hsp80 of *Neurospora,* in extracts of germinating conidia and mycelial homogenates.

References

1. Means, G.A., and Feeney, R.E. 1971. Chemical Modification of Proteins. Holden Day, Inc. San Francisco, Cambridge, London, Amsterdam.

2. Laemmli, U.K. 1970. Cleavage of structural proteins during assembly of the head of Bacteriophage T4. Nature (London) 227:680-685.



Structures of selected homo-bifunctional crosslinking reagents