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Review

Analysis of protein aggregates by combination of cross-linking reactions and chromatographic separations

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Abstract

Chemical cross-linking provides a method that covalently bridges near-neighbour associations within proteins and protein aggregates. Combined with chromatographic separations and protein-chemical methods, it may be used to localize and to investigate three-dimensional relations as present under natural conditions. This paper reviews the chemistry and application of cross-linking reagents and the development of combination experimental approaches in view of chromatographic separations and cross-linking reactions. Investigations of homooligomeric and heterooligomeric protein associations as well as conformational analysis are presented. © 1997 Elsevier Science B.V.

Keywords: Reviews; Cross-linking reactions; Proteins; Protein aggregates

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1. Introduction

The biological functions of almost all proteins are provided by specific, non-covalent interactions with other molecules resulting in the formation of supramolecular aggregates. They are individual in composition, size, subcellular localization and three dimensional configuration. Coordinated protein associations are required: (1) to maintain the biological activity of many enzymes and diverse hierarchic enzymatic cascades; (2) for receptor dependent signalling cascades; (3) for cellular processes such as

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differentiation, growth, migration, apoptosis, mitosis and metabolism; (4) to build the structure and architecture of biological systems at the level of subcellular (cytoskeleton) and higher structures (organization of a multicellular organisms).

Besides intermolecular protein interactions, the three dimensional structure of a given protein as it is determined by near-neighboured, intramolecular associations, plays an additional important role in maintaining its biological activity. As is known from membrane receptors, members of signal transduction pathways and a variety of other proteins, changes of spatial arrangements often result in a transformation of a given protein from one into another activity status. Again, the conformation may be further affected by interactions with lipids, transient covalent modifications like phosphorylation of tyrosine residues or interactions with other proteins. Finally, intramolecular associations may itself regulate intermolecular relations. Information on selected aspects of protein interactions and their biological consequences may be obtained from the references listed in the following [1–15].

In summary, the conformational dependent association of proteins provides not only the molecular basis for their function but represents the basis of molecular communications within cells. Thus, the study of inter- and intramolecular protein interactions should give substantial information about the action of proteins and their implications in biochemical events. The abundant literature dealing with investigations of protein associations demonstrates the significance of this topic.

A range of analytical methods have been developed to investigate protein interactions. First, size exclusion chromatography represents a successful tool to isolate and characterize whole protein aggregates [16,17]. Second, protein aggregates may be analyzed by centrifugation techniques [18]. Third, electrophoretic separations have also been successfully utilized to detect and separate protein complexes [19,20].

The application of immunopurification techniques to the characterization of protein associations displays another variant of experimental techniques [21]. It utilizes immobilized antibodies. If other proteins (protein subunits) are complexed with the corresponding antigen (protein of interest), they can be copurified together with the antigen. This makes immunopurification especially convenient for the search of unknown binding partners.

However, all these approaches also have some disadvantages reducing their potential. One difficulty is related to the presumption that the protein aggregates have to be handled under native conditions. Therefore, artificial protein interactions can hardly be distinguished from that of specific aggregates. On the other hand, purification and analysis of protein aggregates often result in the dissociation of molecular complexes or in the denaturation of their single compounds. Complications of investigation especially concern protein associations of low affinity or short half life and, in particular, interactions of hydrophobic proteins such as membrane-associated or transmembrane proteins.

To overcome these difficulties, experimental approaches have to be chosen which preserve the near-neighbour relationships and molecular associations present in native protein complexes before further steps of purification and analysis are performed. The application of chemical cross-linkers is one concept to realize this requirement. Chemical cross-linkers are group specific, bifunctional reagents with defined molecular sizes. They form covalent bridges if (i) a protein contains chemically reactive side chains and (ii) if the spatial distance between peptide chains in proteins and protein complexes corresponds with the length of the used cross-linker. Once cross-linking has occurred, the modified molecules/molecular complexes keep their original structure and composition even under harsh denaturing conditions. Therefore, the cross-linked protein complexes should be simply susceptible to separation from unmodified molecules without consideration of experimental conditions which preserve native structures. On the contrary, denaturation should improve the separation efficiency of cross-linked complexes from other, unmodified molecules/complexes because these are dissociated into single components. Finally, appropriate protein-chemical techniques should allow the analysis of enriched protein complexes with respect to composition and position of cross-linking sites.

This review will first focus on the chemical mechanism and the application of chemical cross-linkers. The combination of chemical cross-linking

with chromatographic techniques will be discussed in view of designing a combinatorial experimental approach. Finally, the practical importance of this experimental approach will be demonstrated by means of investigations of homooligomeric and heterooligomeric protein associations and with respect to the analysis of intramolecular conformations.

2. Application of chemical cross-linkers

Chemical specificity, length and cleavability of a cross-linker are significant characteristics which have to be considered at the beginning of a cross-linking experiment. In addition, the choice of a cross-linker should depend on the experimental question, i.e. whether inter- or intramolecular cross-linking is the aim of the experiment.

Chemical cross-linking needs two prerequisites: First, the protein(s) must contain chemically reactive side chains. NH_2-, SH- or COOH-residues of amino acid side chains provide convenient targets for selective covalent protein modification. Second, the distance between neighbouring residues within a protein(aggregate) must fit with the molecular size of the cross-linker. Theoretically, chemical cross-linkers are capable to modify proteins in three different modes. As is shown in Fig. 1A, cross-linking with reagents of appropriate sizes might occur: (1) between adjacent proteins (intermolecular); (2) within one peptide chain (intramolecular), which has importance in view of investigations of conformational states of a protein; (3) without molecular bridging, i.e., covalent protein modification occurs without linking with a counterpart.[1]

A wide range of cross-linkers has been developed which fulfil the requirements of chemical selectivity. On the other hand, if desired, cross-linkers with less

[1] See also the review about protein derivatization in this volume.

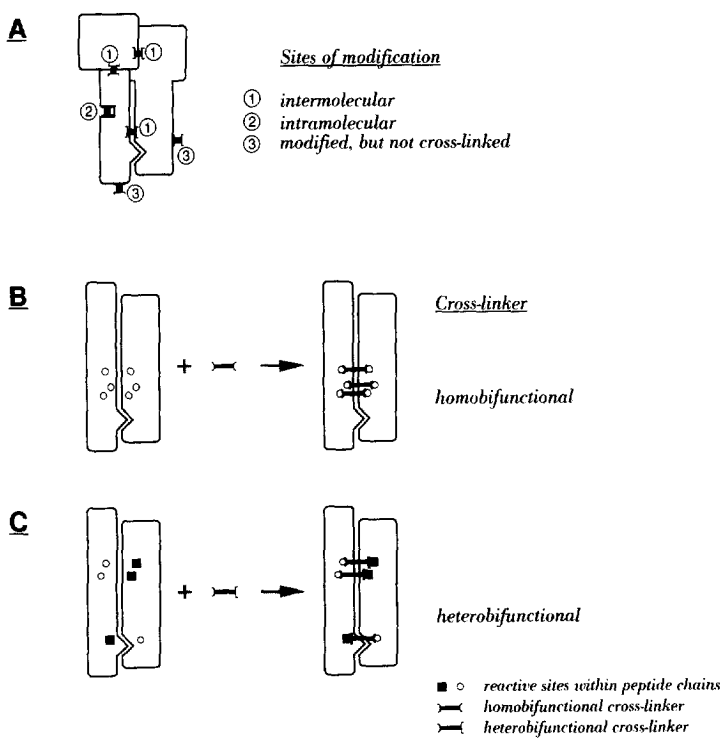
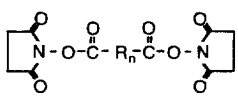
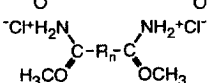
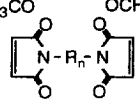
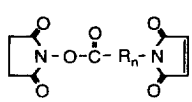
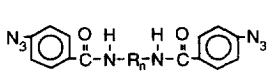


Fig. 1. Principles of protein cross-linking. A, Chemical cross-linking of proteins results in variant sites of modification. B, C Homo- or heterobifunctional cross-linkers may be used to link near neighboured peptide chains within protein complexes.

specificity are also supplied. In general, chemical cross-linking may bridge proteins via amino acid side chains of the same or of different chemical reactivity. Therefore, reagents with the same or with different functional groups have been developed. Cross-linkers with the same chemical specificity are grouped into the class of homobifunctional reagents, whereas heterobifunctional cross-linkers are made of groups of different reactivity (Fig. 1B,C). Table 1 gives a short summary on the molecular structure of some frequently used cross-linkers.

Chemical specificity is one of the most significant property of a cross-linker. To ensure a controlled protein modification at distinct targets within a peptide chain, it is generally best to choose a cross-linker with defined chemical reactivities. In contrast, non selective protein modification enhances the generation of artificially formed aggregates and complicates subsequent analytical procedures. *N*-hydroxysuccinimide esters (NHS-ester) like DST (disuccinimidyl tartarate) [22], DSP (dithiobis(succinimidylpropionate)) [23], DSS (disuccinimidylsuberate) [24] or EGS (ethyleneglycol bis(succinimidylsuccinate)) [25] and imidoesters like DMA (dimethyl adipimidate) [26] or DMS (dimethyl suberimidate) [27] are some homobifunctional cross-linking reagents that react specifically with primary amino groups (usually ϵ-NH_2-groups of lysyl residues), whereas the maleimide BMH (bismaleimidohexane) [28] bridges peptide chains via sulfhydryl groups (Table 1). Examples of heterobifunctional reagents with reactivity towards ϵ-NH_2-groups and sulfhydryl groups are SMCC (succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate) [29], MBS (*m*-Maleimidobenzoyl-*N*-hydroxysuccinimide ester) [30], SMPB (succinimidyl

Table 1
Cross-linkers frequently used in protein-chemical investigations

Type of cross-linker	Structure of active group	Specificity	Target	Examples
Homobifunctional				
N-hydroxysuccinimide ester (NHS-ester)		selective	NH_2-groups	DST (8.6 Å) DSP (12 Å) DSS (11.4 Å) EGS (16.1 Å)
Imidoester		selective	NH_2-groups	DMA (8.6 Å) DMS (11 Å)
Maleimide		selective	SH-groups	BMH (16.1 Å)
Heterobifunctional				
(NHS-ester/Maleimide)		selective	NH_2-groups SH-groups	SMCC (11.6 Å) MBS (9.9 Å) SMPB (14.5 Å)
Carbodiimide (CDI)	R_1-N=C=N-R_2	less selective	NH_2-, COOH-groups	EDC DCC
Arylazide		non selective	electrophilic groups	BASED

NHS-esters, imidoesters and maleimides are supplied with spacers of variable lengths (R_n) between both reactive groups. Homobifunctional cross-linkers contain identical reactive groups; heterobifunctional cross-linkers are composed of different reactive residues. NHS-esters and maleimides are also available in combination in form of heterobifunctional reagents capable to bridge proteins via SH- and NH_2-residues. Carbodiimides are zero-length cross-linkers. BASED is an example of a photoaffinity reagent. It contains two photoactive groups linked by a disulfide spacer. The nomenclature of cross-linkers is listed in the abbreviation section.

4-(*p*-maleimidophenyl)butyrate) [31] or SPDP (*N*-succinimidyl 3-(2-pyridyldithio)propionate) [32].

All these reagents are manufactured with spacers of different sizes and different polarity. Especially the cross-linker's length finally determines the result of a cross-linking approach. Theoretically, a cross-linker with an appropriate size should bridge only molecular distances corresponding to its length. The size of a cross-linker should therefore be chosen in such a way that near neighboured (specific) protein interactions are linked covalently, whilst other molecules which are not in the appropriate distance, remain unmodified. Molecules with intermediate intermolecular distances are bridged by cross-linkers with intermediate size, molecules with long intermolecular distances are bridged by reagents with long spacers. Furthermore, the length of a cross-linker should be suitable to determine spatial distances between the single components of a protein aggregate. On the other hand, so called zero cross-linkers favour intramolecular bridging, i.e., linking of very small distances. However, there are also observations reporting no relations between the size of the cross-linker and the course of molecular bridging [33–37]. If this occurs, cross-linking should be performed with modified experimental conditions to make this reaction reproducible and to prevent artificial bridging.

Reagents containing a cleavable spacer further extend the field of cross-linking applications. Their application offers the advantage of compositional analysis of protein complexes after cross-linking and purification. Convenient cleavage sites are disulfide bridges as present in DSP [23], DTSSP (dithiobis-(sulfosuccinimidylpropionate)) [38], DTBP (dimethyl 3,3'-dithiobispropionimidate) [39], SADP (*N*-succinimidyl[4-azidophenyl]1,3'-dithio)propionate) and sulfo-SADP [40,41]. Disulfide bridges allow a mild reductive dissection (as it is usually performed by dithiothreitol, dithioerythritol or mercaptoethanol in reducing SDS-PAGE) of the linked complex. A well established method for the analysis of cleavable protein cross-links is given by two dimensional SDS gel electrophoresis (2D-PAGE) [42]. Following cross-linking, the mixture of (radioactively labelled) protein aggregates is separated according to their size in the absence of reducing agents (first dimension). Subsequently, the lane of separated aggregates is re-electrophoresed in the presence of reducing agents (second dimension). This treatment cleaves the molecular bridges of cross-linked species. The bands containing un-cross-linked protein will not display changes of electrophoretic mobility in the second dimension after reduction whereas the cross-linked species are dissected into their components represented by newly formed bands. Finally, the comparison of the band pattern of untreated and cross-linker treated samples gives information on the nature of protein associations. Investigations of F1-ATPase [43–45] and studies on ribosomal architecture [46–49] are typical applications of this technique.

Apart from the three classes of NH_2- and SH-modifying reagents described above, glutaraldehyde as a traditional cross-linking reagent should be mentioned. It modifies proteins mainly via NH_2-residues, but, however, it exhibits also reactivity towards OH- and SH-residues. The reagent has been successfully used in protein cross-linking and protein conjugation such as the preparation of antibody–enzyme conjugates (see Refs. [50–52] and references cited therein) and is still used in investigations of protein interactions [53–58]. However, the main disadvantage concerns the ability of glutaraldehyde for self-polymerization in solution [59,60]. The length of glutaraldehyde polymers depends on the pH and raises with increased alkaline pH. In general, the property of self-polymerization makes the reagent difficult to handle; the control of the reaction to achieve an optimal protein cross-linking is therefore critical, and irregular aggregate formation may occur. In this case, glutaraldehyde should be displaced in favour of NHS-esters or imidates as listed above which possess a more defined chemical specificity.

Carbodiimides (CDI, [61], see Table 1 for structure) are suitable reagents if carboxyl groups of proteins are to be utilized for protein cross-linking. The reaction mechanism of CDI consists of a single activation step of COOH-residues. The activated COOH-group reacts spontaneously with NH_2-residues, the second target. In detail, the CDI first converts a carboxyl group from aspartate or glutamate residues into an *O*-acylisourea group which can react further, e.g., with an amino group. In contrast to maleimides or NHS-esters, CDI bridge near

neighboured structures in proteins not via a spacer. They are therefore classified into the group of zero cross-linkers and, in view of protein cross-linking, the reaction is called zero-length cross-linking reaction.

The class of photosensitive cross-linkers represents a particular development of cross-linker. They do not spontaneously link neighboured proteins. Cross-linking is initiated only if the mixture of cross-linker and proteins is illuminated with ultraviolet light (e.g., by use of a simple UV lamp) or a bright camera flash. This property is the most significant advantage of this class of chemicals because cross-linking can be initiated at selected times. When interactions of proteins with short half lives or low affinities are to be investigated, or for ligand–receptor studies, photo cross-linking will provide a suitable alternative technique to common reagents.

Arylazide groups provide the molecular basis of photo cross-linking. Radiation with UV light converts the arylazide residues into reactive arylnitrene groups. Subsequently, the arylnitrenes react with electrophilic groups of amino acid side chains providing a fast, but non selective cross-linking. The reagent BASED (bis-[β-(4-azidosalicylamido)ethyl]disulfide) as shown in Table 1 is one member of the group of photo cross-linkers. It is a homobifunctional substance made of two arylazide groups and a 12 Å long spacer containing a disulfide linkage [41]. In addition to homobifunctional photosensitive cross-linkers, photoaffinity reagents are also manufactured with a residue of other reactivity, e.g., in combination with a NHS-ester group. Examples are SADP (*N*-succinimidyl[4-azidophenyl]1,3′-dithio)propionate) [40], SANPAH (*N*-succinimidyl-6-(4-azido-2′-nitro-phenylamino)hexanoate) [62], HSAB (*N*-hydroxy-succinimidyl-4-azidobenzoate) [63] or SSASDP (sulfosuccinimidyl 2-(4-azidosalicylamido)ethyl-1,3-dithiopropionate) [64]. This class of heterobifunctional photosensitive cross-linkers may be especially applied for investigations of receptor–ligand interactions. First, the ligand is labelled with the photosensitive group via primary amines. The second step consists of ligand–receptor binding. In the third, the ligand–receptor complex is covalently linked by UV radiation converting it into a non dissociable form. Now, the aggregate may be analysed by common protein chemical techniques.

Another advantage of arylazides is that the photosensitive group can be iodinated. This allows the introduction of an additional radioactive label into the cross-linked complex and makes its detection easier. Theoretically, the localization of the incorporated radioactivity within a peptide chain should also be possible.

For comprehensive information on the field of protein cross-linking, the books of S.S. Wong [65], and P. Tijssen [52], as well as the catalogue of the Pierce company (Rockford, IL, USA) are recommended. They give a good survey of the theoretical basis of cross-linker chemistry and of the increasing amount of published cross-linker applications. Also, the monographs contain selected experimental details on protein cross-linking. Another review [42] lists further practical insights into working with disulphide cross-linking and its use in protein analysis.

The fact that proteins vary enormously in amino acid composition, three-dimensional configuration and post-translational modifications requires an individual cross-linking protocol for every investigation. A generally applicable instruction for handling a cross-linking reaction and for choosing a specified cross-linker can therefore not be given. In general, the beginning of a cross-linking experiment should start with the examination of the protein(s) of interest with respect to available reactive sites, i.e., NH_2-residues, free SH-groups and carbohydrate residues. In addition, the subunit composition of a protein and the character of their interaction (covalent or non covalent, low or high affinity) have to be considered. Furthermore, the investigation of membrane or membrane-associated proteins of intact cells or organelles must also take into account whether the cross-linker should be membrane impermeable (water soluble) or membrane permeable (water insoluble). Water soluble cross-linkers usually carry additional sulfo groups, whereas the others have to be dissolved in a polar solvent like DMSO.

Normally, a cross-linking approach starts with a simple trial and error experiment using variable concentrations of cross-linker, reaction times and protein concentrations of the sample. The progress of cross-linking is monitored by SDS-PAGE or SDS-PAGE/immunoblotting; the samples treated with reagent are compared to the untreated control. If cross-linking occurs specifically, the protein(s) of interest should be converted into complexe(s) with

Table 2
Conditions of cross-linking

Reagent	Concentration	Conditions	Reference
BMH	0.1 m*M*	90 s, RT	[70]
	2 m*M*	20 min, 4°C	[101]
DSS/DSP	1–5 m*M*	15–30 min, 4°C	[35,36,90,92]
	1 m*M*	2 h, 4°C+1 h, 37°C	[33]
DMS	37 m*M*	1–20 min, RT	[91]
DTBP	20 m*M*	4 h, 0°C	[49]
EDC	2–20 m*M*	30 min, RT	[84,102]
GA	13 m*M*	20 min, RT	[53]

RT, room temperature.

higher M_r as compared with the non modified protein(s) resulting in distinct additional bands in SDS-PAGE. On the other hand, the appearance of smeared bands indicates that modification was irrelevant. In summary, the following parameters have to be considered in detail: (1) time of reaction; (2) temperature; (3) protein concentration; (4) molar ratio between cross-linker and protein; (5) pH; 6, intensity and time of UV illumination, if a photosensitive cross-linker is used.

Some examples of reaction parameters are listed in Table 2; the relation between the molar ratio of cross-linker and protein is demonstrated in Fig. 2. It should be noted that covalent modifications normally take place within seconds or minutes. Therefore, the cross-linking reaction has to be stopped carefully on time by use of an appropriate quencher which blocks remaining cross-linker molecules. Prolongation of reaction may influence the quantity of covalent dimer, trimer or tetramer aggregate formation of some oligomeric proteins. However, in other cases, it enhances only the number of irrelevant side chain modifications. This affects the surface properties of many proteins and reduces their detection with

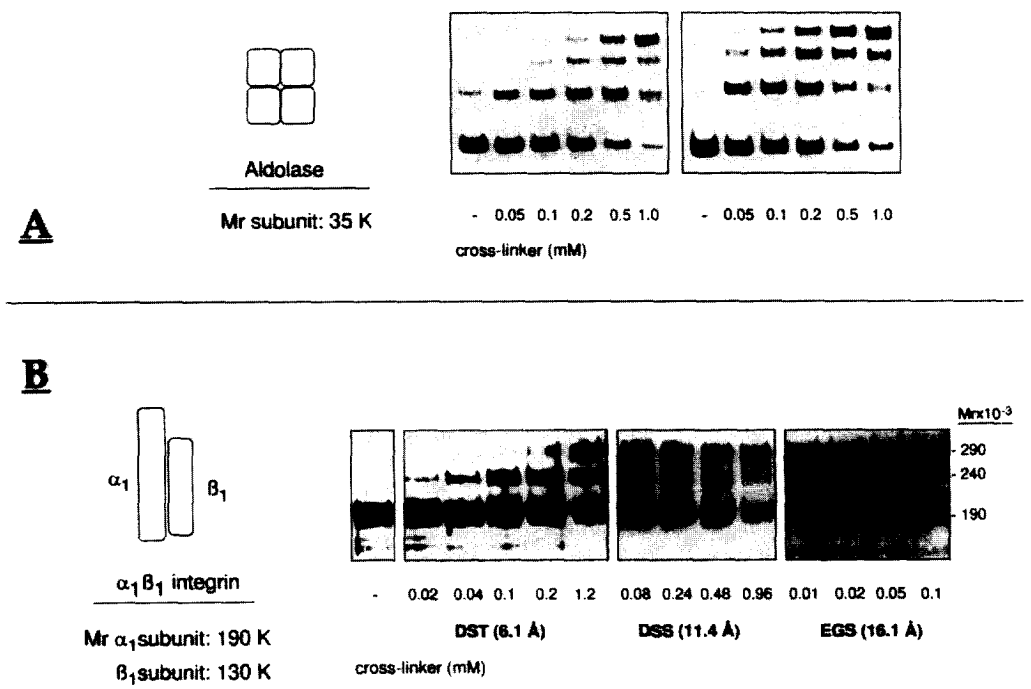


Fig. 2. Influence of cross-linker concentration on progress of aggregate formation. The tetrameric homooligomer aldolase (A, M_r of the monomer: 35 K) and the dimeric heterooligomer $\alpha_1\beta_1$ integrin (B, M_r of the single subunits 190 K and 130 K) were treated with variant reagent concentrations (aldolase: DTSSP (left) or BSS (right); $\alpha_1\beta_1$ integrin: DST, DSS, EGS). Aldolase was visualized by staining with Coomassie blue G-250; $\alpha_1\beta_1$ integrin was detected using polyclonal antibodies against the α_1-subunit [100]. Note that the efficiency of cross-linking into oligomeric aggregates depends on the reagent size and concentration, and that the heterodimeric protein $\alpha_1\beta_1$ integrin is cross-linked into two high molecular mass aggregates with 240 K and 290 K. Cross-linking of aldolase was reprinted from Staros [38], with kind permission.

Table 3
Combination of cross-linking and chromatographic separations: Separate application of chromatographic techniques and chemical cross-linking to verify protein interactions

Application	Interaction	Cross-linker	IEC	AC	SEC	RPC	Ref.
Phosducin: Transducin	heterophil	PPDM	○	○	●	○	[103]
G protein beta: gamma subunit	heterophil	BMH	○	○	●	○	[101]
Thyroid hormone receptor (T3R): T3R auxiliary protein	heterophil	GA	●	○	●	○	[58]
Glucocorticoid receptor	heterophil	DSP	○	○	●	○	[104]
Avian sarcoma virus integrase (ASV IN)	homophil	DSP/BS	○	○	●	○	[104]
Transcription factor heat shock factor (HSP)	homophil	EGS	○	○	●	○	[74]
Human p53	homophil	GA	○	○	●	○	[55,67]
DnaB helicase	homophil	GA	○	○	●	○	[54]
trp repressor (TR)	homophil	EDC	○	○	●	○	[105]
NPY receptor: Neuropeptide Y	heterophil	ANB-NOS*	○	●	○	○	[106]
DNAse I: myosin subfragment 1	heterophil	EDC	○	●	●	●	[107]
Eukaryotic transcription factor TFIID	homophil	BMH	○	○	●	○	[68]
HIV-1 transmembrane envelope glycoprotein gp41	homophil	EGS	○	○	●	○	[108]
Capsid protein p24 of HIV type 1	homophil	DSP/DTSSP	○	○	●	○	[109]
Cytochrome *P*-450scc: cytochrome *b*5	heterophil	EDC	○	●	○	○	[110]
Signal sequence receptor subunits	heterophil	DSP/DTSSP/DMS	○	●	○	○	[111]
RNAse III	homophil	DST	●	○	●	○	[72]
Cell nuclear factor alpha CP1	heterophil	DSP	○	○	●	○	[112]
DNA-binding protein GT1a	homophil	DMS	○	○	●	○	[69]
Transcription factor USF[43]	homophil	GA	○	○	●	○	[56]
IFNγ: IFNγ receptor	heterophil	GA	○	○	●	○	[57,82]
Actin: fragmin	heterophil	EDC	○	○	●	○	[113]
Carbamyl-phosphate synthetase	homophil	DMS	○	○	●	○	[71]
Mitogenic fibrinogen receptor: fibrinogen	heterophil	EGS	○	●	○	○	[80]
Rat mannose-binding protein	homophil	BSS	○	○	●	○	[114]
Citrate synthase	homophil	DMS/GA	○	○	●	○	[115]

Interaction means type of protein–protein interaction; (●) indicates the application of the corresponding technique; * indicates a photoactive cross-linker. The nomenclature of cross-linkers is listed in the abbreviation section.

antibodies in immunoblotting or the purification by AC. Besides, some members of the cross-linker family are extremely sensitive against an aqueous, basic environment (e.g., bisimidates). They hydrolyze within a few minutes.

Finally, the stoichiometry of the components of a protein aggregate and the relation between assembled and non assembled molecules of a given protein within a sample need special consideration. In case of SDS-PAGE/immunoblot analysis of protein cross-links, the disproportional appearance of cross-linked aggregates may interfere with the specific detection of minor cross-linked components. These are represented by faint bands which may be interpreted to be non specific or artificial. It is therefore important to appreciate that a minor band could also be the result of specific cross-linking. If such a complicated course of cross-linking is expected, the application of further analytical or preparative techniques will be of use to distinguish between artificial and authentic complexes.

Detailed information on experimental conditions for using cross-linkers can be obtained from the references listed in Tables 3 and 4 and from the monographs listed above.

3. Methodologies for combination of chemical cross-linking and chromatographic separations

As outlined above, chemical cross-linking freezes defined native configurations and protein associations, and serves as a prerequisite for their detection and analysis. Subsequent experimental procedures cannot change these configurations. Therefore, they shall be susceptible for purification and analysis, and monomers or irregular, artificial complexes can be separated from the others. In combination with the

Table 4
Combination of cross-linking and chromatographic separations: direct application of chromatographic techniques to analyse/enrich cross-linked protein complexes

Application	Interaction	Cross-linker	IEC	AC	SEC	RPC	Ref.
Ferredoxin: NADP reductase	heterophil	EDC	●	○	○	●	[116]
Myelin basic protein	homophil/intra	DSP	○	○	●	○	[76]
Mammalian steroid hormone receptors	heterophil	DMP	○	●	○	○	[117]
Parathyroid hormone: mitochondrial ATPase	heterophil	SSAED*	○	○	○	●	[118]
Anabaena PCC 7119 flavodoxin: ferredoxin–NADP+ reductase	heterophil	EDC	○	○	○	●	[88]
Glucose transport protein (GLUT1)	homophil	GA	○	○	●	○	[53]
HmaL23: HmaL29 (50S ribosomal subunit)[a]	heterophil	DSP, DB	●	○	○	●	[33]
HL29: HL31 (50S ribosomal subunit)	heterophil	DSP	●	○	○	●	[86]
Human fibroblast growth factor receptor (XC-FGF-R): recombinant human bFGF (rhbFGF)	heterophil	DSS/BS/DTSSP/ BSOCOES	○	○	●	○	[36]
TATA-binding protein (TBP): c-*Myc* oncogen	heterophil	DSP	○	○	●	○	[90]
Neuropeptide Y (NPY): receptor NPY2	heterophil	DSS	○	○	●	○	[92]
ras binding proteins	heterophil	DSS/EGS	●	●	○	○	[79]
Troponin C (TnC): Inhibitory protein I (TnI)	heterophil	EDC	○	○	○	●	[119]
Bovine hemoglobin	intra	NFPLP	○	○	○	●	[93]
Interleukin-1 beta	intra	EDC	○	○	●	●	[97]
$\beta_1\alpha_6$ integrin: calnexin	heterophil	DSP	○	●	○	○	[78]
Porcine/ovine luteinizing hormone (pLH, oLH)	homophil	EDC	○	○	●	●	[75]
Human granulocyte-colony stimulating factor (G-CSF): G-SF receptor	heterophil	DSS	○	○	○	●	[120]
Rat $\alpha_1\beta_1$ integrin	heterophil/intra	DSS/DSP	○	●	●	○	[35,98]
Human $\alpha IIb\beta_3$ integrin	heterophil/intra	DSS/DSP	●	○	○	○	[66]
Porcine ribonuclease inhibitor (RI)	intra	DTNB	○	○	●	○	[121]
Gizzard calponin:actin	heterophil	EDC	○	●	○	●	[84,85]
Mitogenic fibrinogen receptor: fibrinogen	heterophil	EGS/DSP	○	●	○	○	[80]
Escherichia coli heat-stable enterotoxin receptor: enterotoxin	heterophil	EDC	○	○	●	○	[122]
Bacteriophage T4 baseplates	heterophil	EGS	○	○	●	○	[114]
Rat liver 80 S ribosomes	heterophil	IT	●	○	○	○	[123,124]
60 S ribosomal subunits	heterophil	DTBP	●	○	○	○	[49]
Cytochrome *c*: plastocyanin	heterophil	EDC	●	○	○	○	[125]
Myosin subfragment I light: heavy chain	heterophil	DSS/DSP	○	○	○	●	[126]
Phosphoribulokinase	intra	DFDNB/FNPS	○	○	○	●	[96]
T7 DNA primase: helicase	heterophil	DMS	○	○	●	○	[91]
Interleukin-1 beta	intra	EDC	○	○	●	●	[97]
HMG proteins 14/17: histone H3	heterophil	SAPDP*	○	○	○	●	[127]
Beta subunit of F1-ATPase[a]	homophil	EDC	○	○	○	●	[44]
G-actin: myosin subfragment-1	heterophil	PPDM/EDC	●	○	●	○	[37]
TATA binding protein	homophil	BMH	○	●	●	○	[70]
Cytochrome *c*: cytochrome *b5*[a]	heterophil	EDC	●	○	●	●	[102]
Vitronectin receptor: RGD	heterophil	SSASDP*	○	●	○	●	[87]
Lysozyme	intra	BBA	●	○	●	●	[94,95]
Lectin: lectin receptor	heterophil	SANPAH/HSAB*	○	●	○	○	[62]

Interaction means type of protein–protein interaction; (●) indicates the application of the corresponding technique; * indicates a photoactive cross-linker.

[a] Details on chromatographic separations are listed in Table 5. The nomenclature of cross-linkers is listed in the abbreviation section.

advantages of chromatographic techniques, chemical cross-linking should offer the possibility to obtain detailed information on tertiary and quaternary structures of proteins and protein complexes. On the one hand, common chromatographic separations like IEC, AC or SEC may be used to purify cross-linked

proteins; on the other hand, SEC or RPC are reliable tools for further analytical steps, e.g., if peptide mapping of purified complexes is necessary.

In practice, the development of a combinatorial approach may contain variant strategies. Fig. 3 gives an schematic review of the development of a combinatorial approach. At the beginning, the source (membrane, organelles, cells, secretory or cytosolic proteins) and the solubility of the protein aggregate of interest have to be considered. If the proteins are insoluble, one of the subsequent steps must include protein extraction with detergents. Furthermore, the question has to be answered, whether the protein aggregate should be partially purified before cross-linking (this might affect native interactions) or whether cross-linking has to be performed first. The sequence of the next purification and analytical steps may combined as shown in Fig. 3. Unfortunately, in most cases, the optimal way to solve the complete task of analysis of protein interactions cannot be predicted. The whole approach has to be improved to find out the appropriate experimental way.

In addition, the choice of methods to detect the protein of interest and the progress of cross-linking needs special consideration. SDS-PAGE represents

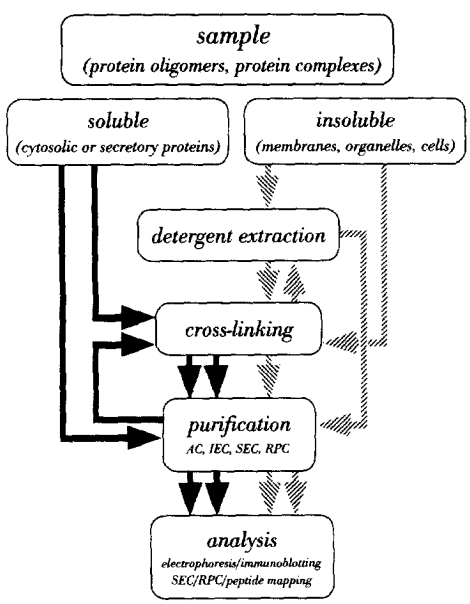


Fig. 3. Investigation of protein–protein interactions by combination of chemical cross-linking and chromatographic separations: scheme of experimental steps.

an easy and fast way to obtain an overall picture of cross-linking. It has been applied in almost all references cited in Tables 3 and 4. If the protein aggregate is purified, a simple protein stain may be used. The cross-linked sample moves as band(s) with higher M_r as compared with the unmodified protein(s). In case of crude protein mixtures or less quantities of protein, immunoblotting will probably provide the better and more specific method of detection. In some cases, however, immunoblotting will be complicated because the covalent modification has changed the antigenic properties of the protein [66].

4. Applications of cross-linking reactions and chromatographic separations in the analysis of protein associations

A scan of Medline (1986–1997) results in a huge number of applications concerning the investigation of protein associations by using various chemical cross-linkers. Most of them apply chemical cross-linking to look for selected proteins within more or less hierarchically structured aggregates of soluble or membrane proteins. However, a small number of papers go into detailed investigations of protein associations. A part of these contain applications of cross-linking reactions in combination with chromatographic separations.

First, cross-linking serves to confirm the existence of a given protein in complexes which were primarily detected by other biochemical methods, e.g., SEC or centrifugation (sedimentation analysis). In combination with denaturing electrophoretic techniques, usually SDS-PAGE, the given protein is visualized by staining, autoradiography or immunoblotting within newly formed high molecular mass band(s) as compared to a non cross-linked sample. Table 3 summarizes references which make use of chemical cross-linking to confirm data on protein aggregates obtained by chromatographic separations. Second, cross-linking serves to identify unknown protein(s) acting as binding partners to known components. This special task uses cross-linking to freeze intermolecular associations which normally dissociate during purification. Once the complex has been cross-linked, chromatographic separations are ap-

plied to purify the aggregate(s), which may subsequently be cleft to identify single components. Examples of this application are listed in Table 4.

The third and most interesting approach deals with the molecular localization of cross-linking sites. It is based on the assumption that chemical cross-linking of proteins occurs at distinct locations. Their analysis should therefore provide direct information on near neighboured residues within proteins and protein–protein aggregates. This experimental task utilizes the whole spectrum of chromatographic techniques including purification of cross-linked aggregates, peptide mapping and peptide purification. Finally, N-terminal sequencing gives specific information on the localization of cross-linking and allows the interpretation of individual configurations within a protein complex. A selection of examples is given in Table 4.

All these approaches have been used for investigations of homooligomeric and heterooligomeric protein associations and for intramolecular analysis. They are discussed separately in the following paragraphs.

4.1. Analysis of homooligomers

Investigations of homophilic protein associations using a combination of chemical cross-linking and chromatographic separations include mostly DNA-binding proteins: transcription factor USF[43] [56], DnaB helicase [54], p53 [55,67], transcription factor TFIID [68], DNA-binding protein GT1A [69] and TATA binding protein [70]. In addition, some applications concern enzymes like carbamyl-phosphate synthetase [71] and RNAse III [72] or members of other protein classes: rat mannose binding protein [73] and *Drosophila* heat shock factor [74].

In general, the stoichiometric composition ($n=2$, 3, 4,...) of homooligomers simplifies the prediction of aggregate sizes and enables a distinct classification of the oligomerization status (dimers, trimers, tetramers, etc.). Thus, chemical cross-linking should generate aggregates with molecular masses in SDS-PAGE which correspond to multiple masses of the monomers. Typical examples of investigations are cross-linking of DnaB helicase with GA [54], dipeptidylpeptidase IV with DSP [34] or RNAse III with DST [72].

In this context it is necessary to remember that the cause and efficiency of cross-linking depends both on the reaction time and on the concentration of the cross-linker. The influence of both parameters has been well documented for cross-linking of mannose-binding protein MBP-A from rat [73] and DnaB protein [54]. By studying C-terminal fragments of MBP-A, a Ca^{2+} dependent animal lectin with homotrimeric composition, it was found that increasing concentrations of the cross-linker BSS (bis(sulfosuccinimidyl)suberate) enhance trimer formation whereas descending molar amounts of BSS preferentially induce the generation of dimers. The influence of reaction time on cross-linker induced aggregate formation is illustrated by cross-linking of DnaB protein with GA. Natural DnaB helicase is a homooligomer consisting of six 5.2×10^4 rel. mol. mass units monomers. As shown by SDS-PAGE, addition of GA to the protein generates within the beginning of the reaction a DnaB dimer with approx. 9.7×10^4 rel mol mass units, followed by the formation of trimers and, at prolonged reaction times, oligomers of higher stoichiometry are formed.

Among the papers dealing with the analysis of homooligomeric proteins, the technique of chemical cross-linking has been mainly used to confirm results from gel permeation and sedimentation analysis experiments showing the existence of protein aggregates of variable sizes (Table 3). In most chromatographic applications, the proteins of interest were characterized using SEC and buffer systems which maintain native protein structures/associations. Examples of stationary phases for SEC are Sepharose CL-4B [69], Sephacryl S-300 [71], TSK-gel 3000 [67], Superdex 200 [54,56], Superose 6 HR [74] or G3000 SW [73]. Among these references, the investigation on *Drosophila* heat shock factor (HSF) [74] shows how SEC and sedimentation analysis may be combined with chemical cross-linking (Fig. 4). First, the existence of native HSF within a monomeric (unshocked protein) and a trimeric (heat shocked) conformation was calculated from SEC and centrifugation and verified later by chemical cross-linking of both protein variants with EGS. Interestingly, the assembly of HSF into a homotrimer was found to be not the result of a simple association between three HSF molecules but rather accompanied by conformational changes of the monomers.

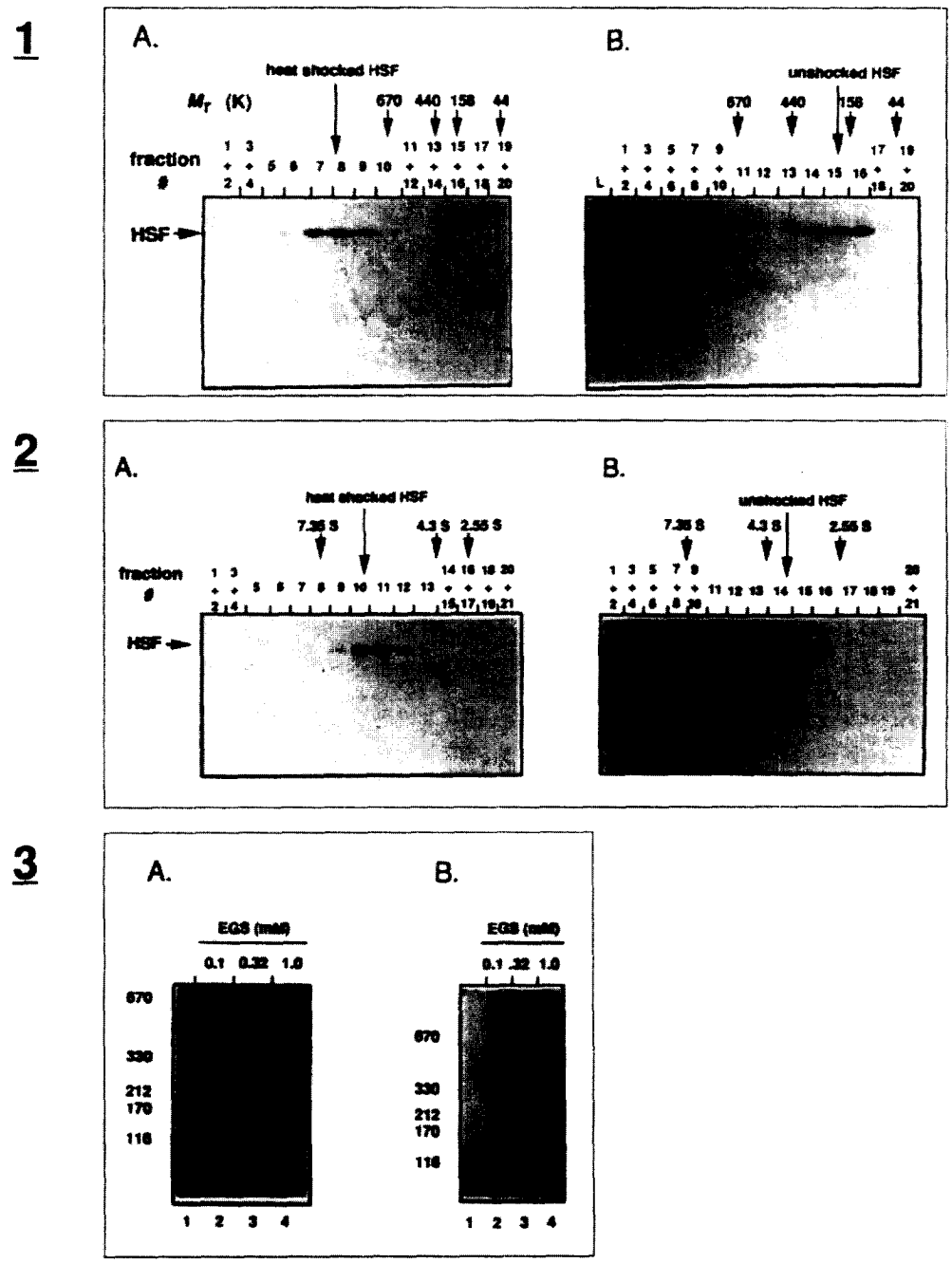


Fig. 4. Analysis of *Drosophila* heat shock factor (HSF) assembly (reprinted from Westwood and Wu [74], with kind permission). (1, 2) SEC (1) and sedimentation analysis (2) of heatshocked (A) and unshocked (B) HSF. SEC was carried out with a Superose 6 column; sedimentation analysis was performed using a glycerol density gradient. (3) Chemical cross-linking of heatshocked (A) and unshocked HSF (B) with EGS. Samples were taken from fractions separated by SEC on a Superose 6 column. Detection of HSF was performed by immunoblotting with polyclonal rabbit anti-*Drosophila* HSF serum.

A correlation between conformation and oligomerization was also observed for p53, a DNA-binding protein with regulatory functions in cell growth. Analysis of size and structure of the purified protein by combination of chemical cross-linking, SEC and gradient centrifugation revealed that the homotetrameric form of p53 represents an aggregate with an elongated rather than spherical shape [67].

Besides these investigations, the studies on porcine luteinizing hormone [75], myelic basic protein [76], β_2 subunits of F_1-ATPase [44] or glucose transporter Glut1 [53] may be grouped within the second experimental strategy (Table 4) wherein chromatographic separations have been directly combined with chemical cross-linking to study arrangements of homooligomeric proteins.

The description of structural motifs involved in the interaction of β_2 subunits of mitochondrial F_1-ATPase represents an interesting example of a combinatorial approach [44]. The experimental sequence applied in this paper represents a general scheme for investigations of protein associations. Details of chromatographic separations are listed in Table 5. In the beginning, the protein was radioactively labelled by adding ^{14}C-DFDNB (1,5-difluoro-2,4-dinitrobenzene) to a suspension of F_1-ATPase followed by reduction of introduced nitro groups to amino groups with sodium hydrosulfite. In the next step, cross-linking was initiated by mixing the protein solution with EDC. Then, the cross-linked subunits were denatured with guanidine HCl and separated by RPC. The β_2 enriched sample was subsequently cleaved into fragments with pepsin. A sequence of further RPC separations served to purify single peptides. Among them, one radiolabelled component was isolated. Finally, this fragment was analyzed by N-terminal sequencing. It contained the phenylendiamine bridged dimer of the tetrapeptides KTVL and YMEM from two β-subunits. From this result and other findings the authors conclude that the quaternary structure of mitochondrial F_1-ATPase is characterized by a triangular set of three β-subunits that sits above a triangular set of three a-subunits in a staggered conformation.

A similar experimental strategy is described by van Dijk and Ward [75]. They have combined EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) cross-linking with SEC and peptide mapping to study the interaction between the subunits of luteinizing hormone (LH). The cross-linked LH was purified by chromatography on Sephadex G-75, digested with endoproteinase Arg-C and the peptide fragments were sequenced. Finally, they were able to determine the position of the cross-link between alpha-Lys49 and beta-Asp111 which are exposed at the subunit–subunit interface.

As mentioned above, denaturing SEC may also permit the detection and isolation of cross-linked protein aggregates. These remain assembled, whereas denaturation dissociates non cross-linked oligomers. Hebert and Carruthers [53,77] and Caamano and Zand [76] used this technique to study glucose transporter (Glut1) and myelin basic protein (MBP). In case of Glut1, the GA cross-linked protein has been gel separated in the presence of SDS and compared to the elution behaviour of the untreated protein. In addition with substrate binding experiments, the transporter was found to represent a homotetramer in which subunits interact cooperatively. Its native structure is stabilized by intrasubunit disulfide bonds that are necessary for structure and function of the transporter.

Examples of the investigations of homooligomeric proteins are summarized in Tables 3 and 4.

4.2. Analysis of heterooligomers

The exploration of heterooligomeric protein associations turns out to be a more complicated task than the investigation of homooligomers. One reason lies in the stoichiometric relation of protein complexes which remains disproportional in a lot of cases. This makes the prediction of aggregates formed by chemical cross-linking difficult. The search for binding partners/effector proteins of a given protein needs special consideration of possible large disproportional stoichiometries between the components of a protein aggregate with molar ratios of 1:10 and higher. Second, most physiologically relevant cellular protein associations need the participation of only some molecules of a given protein in interactions with others, whereas the majority of the molecules remain non-assembled. Third, heterooligomeric protein associations with functions in signal transduction and regulation of cellular

Table 5
Combination of cross-linking and chromatographic separations: examples of experimental conditions to the analysis of protein interactions

Ref.	Application		Stationary phase	Mobile phase/elution mode
[33]	Purification of cross-linked proteins	IEC	DEAE-cellulose DE 52	Gradient (a) 80 m*M* Tris citrate, 4 *M* urea pH 8.3 (b) 300 m*M* KCl in (a)
		RPC	Vydac C_4	Gradient (a) 0.1% TFA in water (b) 0.1% TFA in 2-propanol
	Peptide mapping (LysC, ChTr, TL)	RPC	LiChrospher RP18	Gradient (a) 0.1% TFA in water (b) 0.1% TFA in ACN
[44]	Purification of cross-linked proteins	RPC	Vydac C_4	Gradient (a) 30% ACN in water–0.1% TFA (b) 50% ACN in water–0.1% TFA
	Peptide mapping (pepsin)	RPC	PEP RP1	Gradient (a) 0.1% morpholine, 0.125% TFA in water (b) 0.1% morpholine, 0.125% TFA in ACN
			C_{18} NovaPak (Tandem)	Gradient (a) 0.1% morpholine, 0.125% TFA in water (b) 0.1% morpholine, 0.125% TFA in ACN
[84,85]	Purification of cross-linked proteins	AC	Immobilized Calmodulin	isocratic 1 m*M* EGTA, 200 m*M* KCl, 0.1 m*M* DTT in20 m*M* imidazol–HCl pH 7
	Peptide mapping (BrCN)	RPC	Aquapore C_4	Gradient (a) 0.1% TFA in water (b) 95% ACN in (a)
[102]	Purification of cross-linked proteins	SEC	Sephadex G75	20 m*M* sodiumphosphate pH 7.2
		IEC	MonoQ HR5/5	Gradient (a) 20 m*M* triethanolamine pH 7.3 (b) 300 m*M* NaCl in (a)
		IEC	MonoS HR5/5	Gradient (a) 20 m*M* sodium phosphate pH 7.2 (b) 200 m*M* NaCl in (a)
		IEC	Cm-cellulose	Gradient (a) 10 m*M* Mes pH 5.6 (b) 10 m*M* Mes pH 6.1/ 10 m*M* Mes pH 7
	Peptide mapping (Tr, BrCN, SM)	SEC	Sephadex G100 SF	0.05% TFA in water
		RPC	C_4	Gradient (a) 10% ACN in water 0.05% TFA (b) 50% ACN in water 0.05% TFA
		RPC	C_{18}	Gradient (a) 0.05% TFA in water (b) 75% ACN in (a)

ChTr=chymotrypsin; LysC=endoproteinase Lys-C; Tr=trypsin; V8=endoproteinase Glu-C from *Staphylococcus aureus* V8; SM=mouse submaxillarus protease; TL=thermolysin; ACN=acetonitrile; BrCN=cyanogen bromide; TFA=trifluoroacetic acid.

metabolism are often characterized by short half lives and low affinities.

Similarly to investigations of homooligomeric proteins, heterooligomers have been studied both with direct and with separate applications of chromatographic separations and cross-linking techniques (Tables 3 and 4). Besides IEC which has been used to purify cross-linked species (see Table 4), a number of references applied immunopurification techniques. If covalent protein modification by a cross-linking agent does not have influence on antibody-binding, the cross-linked complex can be purified to homogeneity out of a crude starter sample. Similar to 2D-PAGE, the application of cleavable cross-linkers allows the compositional analysis of purified cross-linked species. In addition to the extreme selectivity of IAC in protein purification, the high recovery permits the enrichment of smallest amounts of a cross-linked protein aggregate. In addition, the freezing of native protein interactions by chemical cross-linking excludes possible dissociation of protein–protein aggregates during preparation and allows the application of harsh washing conditions.

Investigations on mouse $\beta_1\alpha_6$ integrin [78] or the GTP-binding protein ras [79] represent examples for the search of minor heterooligomeric protein interactions combination of chemical cross-linking and immunopurification techniques. In the case of $\beta_1\alpha_6$ integrin, the detergent extract of a mouse endothelial cell line was applied to an anti β_1-chain immunoaffinity matrix. The retained protein was eluted and analyzed by reducing SDS-PAGE. A faint additional band at 9.0×10^4 rel. mol. mass units was copurified and identified by N-terminal sequencing as calnexin, a membrane-bound chaperone and protein of the endoplasmic reticulum. To verify the native character of this interaction, the same experiment was made with a DSP-treated detergent extract of endothelial cells. Reductive cleavage of the immunopurified material showed the same protein composition as it was observed without cross-linking. Based on further data obtained from combination of cross-linking with pulse-chase experiments, the authors conclude that the chaperone calnexin is involved in the subunit assembly of β_1 integrins. The search for ras-binding proteins [79] started with iodination of ras, followed by mixing of ^{125}I-ras with whole cytosolic protein preparations. Cross-linking with EGS, partial purification of the mixture on DEAE cellulose and immunopurification with an anti ras-immunomatrix identified a radioactive labelled complex of 8.5×10^4 rel. mol. mass units. Treatment of the immunoprecipitate with hydroxylamine cleaved the complex, and two ras binding proteins with 6.5×10^4 rel. mol. mass units and 2.0×10^4 rel. mol. mass units were detected.

The identification of a mitogenic fibrinogen receptor on the hemopoietic cell lines Raji and JM represents another typical experimental approach to search for ligand–receptor interactions consisting of cross-linking and immunopurification [80]. The method includes incubation of ^{125}I-surface labelled cells with the ligand of interest (fibrinogen), in vivo cross-linking of the ligand to the cell surface using a bifunctional cross-linker (EGS and DSP) followed by detergent extraction of membrane proteins and immunopurification with anti-ligand antibodies (anti-fibrinogen) immobilized to a stationary phase (Sepharose). Finally, the enriched ligand–receptor complex is analyzed by SDS-PAGE/immunoblotting after cleavage of the cross-link. In this investigation, an additional radioactive labelled band at $9.2–9.4\times10^4$ rel. mol mass units was found. Because the M_r of this band did not correspond to the M_r of known fibrinogen receptors the authors concluded that fibrinogen was specifically linked to an unknown cell surface receptor with different characteristics.

Vice versa, investigations on ligand–receptor interactions may be started with radioactive labelling of the ligand, followed by incubation with a sample containing the receptor. In the next step, the sample is treated with a cross-linking agent, and further analytical and purification steps are performed as described above. Examples of this approach are given by studies on human γ-interferon (IFNγ): human IFNg receptor [57,81,82]). The work of Greenlund et al. [57] represents an example on how chemical cross-linking may be applied to confirm data obtained from SEC. Complexes consisting of variant molar ratios of ^{125}I-IFNγ/IFNγ receptor extracellular domain (ECD) were analysed by HPLC–SEC using a SEC-250 HPLC gel permeation column. The stoichiometry of the major ECD–ligand complex was determined with 2 mol ECD per 1 mol of IFNγ suggesting that IFNγ induces receptor

dimerization. This phenomenon was further studied in vivo with a human adenocarcinoma cell line that expresses active IFNγ receptors. The cells were preincubated with excess amounts of ^{125}I-IFNγ, treated with EDC/Sulfo-NHS, lysed and analyzed by SDS-PAGE. When treated with sufficient amounts of cross-linker, a 2.4×10^5 rel. mol. mass units band was observed that resembled the sum of the molecular masses of two receptor molecules and one ligand molecule. Both findings document that IFNγ induces dimerization of its receptor which represents a unique mechanism in cytokine receptor signalling [83].

In addition to IAC, other affinity matrices have been used to enrich protein complexes after cross-linking using AC. In case of investigations on calponin [84] and caldesmon [85], two actin- and Ca^{2+}-calmodulin-binding proteins, calmodulin–Sepharose was used to isolate calmodulin-binding proteins after cross-linking of a partially purified sample with EDC and subsequent digestion with CNBr. The CNBr-digest was loaded onto the column equilibrated with a Ca^{2+}- Mg^{2+}-, DTT- and KCl-containing imidazole–HCl buffer. The elution was accomplished with EGTA instead of $CaCl_2$. Obviously, covalent modification did not changed the calmodulin-binding activity of the investigated proteins. In the next, the single components of the retained total CNBr-digest were purified by RPC and characterized by N-terminal sequencing. It provided evidence that cross-linking occurred between the N-terminal region of calponin (residues 326–355) and the C-terminal segment (residues 52–168) of actin [84]. In contrast to binding of caldesmon and troponin I, the calponin-binding site within actin is characterized by the presence of non-acidic residues and is shown to be a unique interactive region within the molecule. Details on the applied chromatographic separations are listed in Table 5.

Interestingly, Bartegi et al. [85] reported a decrease of the calmodulin-binding activity of caldesmon when cross-linked to calmodulin by EDC. Moreover, the cross-linked caldesmon had lost other properties like inhibition of actomyosin subfragment 1 ATPase and F-actin-binding. The authors therefore hypothesize that the calmodulin-binding region of caldesmon which was characterized by N-terminal sequencing, contains major regulatory determinants of caldesmon. However, a possible inactivation of caldesmon as a result of covalent protein modification during cross-linking is not discussed.

Another combination of immunopurification and cross-linking is given by the application of anti cross-linker antibodies [62]. These antibodies recognize molecular structures located within the backbone of the cross-linker's spacer. Because a cross-linking agent represents a small hapten without immunogenic properties, it must be coupled to a carrier protein to induce an antibody response. Immobilized to a stationary phase, the antibody can be utilized in the purification of cross-linked protein–protein aggregates. This technique was taken to isolate a concanavalin A (Con A): Con A-receptor complex from erythrocyte membranes. Polyclonal rabbit antibodies were raised against SANPAH and HSAB coupled to bovine serum albumin (BSA) and purified by IAC using SANPAH–BSA and HSAB–BSA–Sepharose. The purified antibody was then coupled to Protein A–Sepharose. Finally, the immunomatrix served for the purification of cross-linker modified protein complexes generated by SANPAH/HSAB-treatment of erythrocyte membranes. Despite the simplicity of this method, the generation and availability of cross-linker specific antibodies proves to be a limiting factor making this technique not generally viable.

Once the cross-linked protein aggregate of interest has been purified, it may be analyzed by further protein-chemical methods. In most cases, the aim of investigations is addressed to the location of cross-links. A general scheme of analysis (for details see Refs. [33,86–88]) consists of a series of proteolytical or chemical fragmentation combined with fragment purification. For the separation of proteins and peptides, common modes of separation (IEC, RPC) are used (Table 5). The final purification yields single or cross-linked peptides accessible for N-terminal sequencing.

Bergmann et al. [33] give an example of near-neighboured analysis of heterooligomeric proteins representing an experimental strategy to the combinatorial analysis of protein associations in general. In this report, the protein pair HmaL23–HmaL29 from the 50S ribosomal subunit of archaebacterium *Haloarcula marismortui* was studied (for details on chromatographic separations see Table 5). Purified

50S ribosomal subunits were cross-linked with DSP and DB and at first analyzed by 2D-PAGE. Cross-linked HmaL23–HmaL29 pairs appear as major additional spot compared to the regular protein pattern of untreated ribosomal subunits. Interestingly, the amount of HmaL23–HmaL29 cross-linking with DSP and DB was comparable. A further two-step chromatography was applied to purify the cross-linked protein pair. First, the total protein from cross-linked ribosomes was fractionated on a DEAE-cellulose DE52 column. The elution of bound proteins was achieved with a KCl-gradient; eluted proteins were desalted and concentrated by RPC [89]. Approximately 60–70% of contaminating protein could be removed. In the next step, a re-chromatography of the prefractionated sample was performed by RPC using a C_4 column. This purification yielded the purified protein pair. The localization of the cross-linking sites was determined by enzymatic fragmentation of the cross-linked proteins and N-terminal sequencing of purified peptides. To limit the number of peptides, proteolysis was performed with lysylendoproteinase (LysC). The peptide mapping was made on a LiChrospher C_{18} column and compared to the elution pattern of a sample treated with β-mercaptoethanol. Remarkable, only one cross-linked peptide was found. It was subjected to N-terminal sequencing. Only one sequence spanning the region of Ala-52 to Glu-70 from HmaL29 was obtained, whereas no peptide of HmaL23 was found. The authors therefore concluded, that the N-terminus of the HmaL23-peptide is part of the cross-linking site. Another peptide mapping of the original cross-linked LysC peptide with chymotrypsin was made but, however, no further sequence information on the HmaL23-peptide was obtained. Finally, the missing sequence information could be determined by use of plasma desorption mass spectrometry. Ser-1 of HmaL23 was found to participate in cross-link formation.

Another technique for the investigation of heterooligomeric protein associations is photoaffinity ligand cross-linking. It is based on two steps – (i) binding of the ligand (carrying a photoreactive group(s)) to its receptor and (ii) cross-linking of the ligand by photoactivation. Vice versa, the same experiment may also be performed with photoaffinity labelled receptor molecules, if the size of the ligand approximates that of the receptor and if it contains sufficient reactive sites. However, the main criteria of this technique is that derivatization does not change the surface of the molecules so that there is no or only diminished binding between ligand and receptor. In the report of Smith and Cheresh [87], the ligand (Arg–Gly–Asp) binding domain of the vitronectin receptor ($\alpha_v\beta_1$ integrin) was examined, a heterodimeric transmembrane adhesion receptor and member of the integrin family. A ^{125}I-labelled ligand peptide (GRGDSPK) was modified with the photoactive cross-linker SSASDP and incubated with partially purified vitronectin receptor. Following UV radiation, the receptor was analyzed by SDS-PAGE. The ^{125}I-label was found to be mostly associated with the β-chain of the receptor. To confirm the location of peptide incorporation, the radiolabelled β-chain was purified to homogeneity using preparative SDS-PAGE and cleaved with endoproteinase Arg-C (*S. aureus* V8 protease). N-terminal sequencing of the main radioactive labelled fragment revealed that the amino acid residues 61–203 are proximal to the Arg–Gly–Asp binding domain of the β-chain of vitronectin receptor.

Among the investigations of heterooligomeric protein associations (Tables 3 and 4), the separation of cross-linked protein aggregates by SEC should be mentioned. Examples are basic fibroblast growth factor (bFGF): bFGF-receptor [36], c-*myc* oncogene: TATA binding protein [90], T7 DNA primase: helicase [91] and neuropeptide Y (NPY): NPY receptor [92]. Protocols are similar to those obtained with separations of non cross-linked proteins. Examples of stationary phases are SW3000G [92], Bio-Sil SEC 400 [91], Sephacryl S-300 [90] or Superose 6 HR10/30 [36].

4.3. Intramolecular cross-linking

Intramolecular cross-linking (see Fig. 1) is shown to be a special kind of chemical cross-linking. If intramolecular bridging occurs within distinct sites of a given protein, it should allow the determination of near neighboured domains within one protein providing information on the conformational status. In case of oligomeric proteins, intramolecular cross-

linking may also be accompanied by intermolecular bridging. Thus, both aspects of cross-linking has to be considered in the interpretation of analytical data. However, the sites of cross-linking can be prepared similarly as it is performed for investigations of homo- or heterooligomers. As listed in Table 4, some references describe in detail the molecular analysis of protein conformations using the combinatorial approach consisting of chemical cross-linking and chromatographic separations. They include hemoglobin [93], lysozyme [94,95], phosphoribulosekinase [96] or interleukin-1 beta [97].

The study of Brandes et al. [96] deals with the question whether the Calvin cycle enzyme phosphoribulokinase (M_r of the monomer: 40×10^3) shows conformational flexibility of its regulatory sites. To answer this question, the purified enzyme has been treated with the bifunctional, SH-reactive reagents FNPS and DFNB. In the next step, remaining SH groups were carboxymethylated with ^{14}C-iodoacetamide. Then, the protein was digested with trypsin and tryptic peptides were separated by RPC on a LiChrosorb RP8 column and by use of IEC. Edman degradation of FNPS- and DFNB-labelled peptides identified the cysteine residues 16 and 55 to be cross-linked. Both of them are located in the active site region. Consistent with additional data obtained from oxidation/reduction of the enzyme the authors conclude that the intrasubunit distance between the SH-residues of Cys16 and Cys55 is dynamic rather than static.

As mentioned above, zero-length cross-linking of proteins, e.g., with carbodiimides or cross-linking with reagents containing a short spacer favours the bridging of near neigbour distances within proteins. Yem et al. [97] and Yamada et al. [94] applied EDC for cross-linking of lysozyme and recombinant interleukin-1 beta (rIL1β) and analyzed the modified proteins with peptide mapping. In case of rIL1β, the position of cross-linking was determined between Glu111 and Lys138. Interestingly, the bioactivity of the cross-linked protein was retained as compared with the untreated, native sample. On the other hand, the digested (endoproteinase Lys C) cross-linked protein had lost its bioactivity compared with the undigested cytokine. The authors speculate that the structural integrity surrounding the cross-linked region may be crucial for the biological activity of rIL1β. Within lysozyme, application of EDC in the presence of imidazole induced the conversion of the salt bridge between Lys13 and Leu129 into an amide bond [94]. Remarkably, the location of these amide bond fitted with previous results from X-ray crystallographic studies of lysozyme. The authors conclude that the applied method may be in general useful for investigations of intra- or intermolecular salt bridges in contact areas of protein side chains.

Ueda et al. [95] reported on the systematic application of cross-linkers with varying lengths in the investigation of structural conditions of proteins. The studies were done with bis(bromoacetamide) derivatives ($BrCH_2CONH(CH_2)_nNHCOCH_2Br$) containing modified spacers of $(CH_2)_n$ (n=0,2,4,6). In investigations of lysozyme, it was found that the length of cross-linker determines the quality of molecular bridging, i.e., whether intra- or intermolecular cross-linking occurs. Estimation of modification of all lysozyme derivatives by gel filtration with Sephadex G-100 showed that the shortest reagent did not cross-link lysozyme intramolecularly, but generated modified monomers and species of intermolecularly linked dimers, whereas the $(CH_2)_2$ reagent induced intra- and intermolecular bridging. In contrast, the reagents with longer spacers preferentially induced formation of one intramolecular bond. By use of peptide mapping and amino acid analysis the cross-link was located in the direction from His15 to Lys1. Furthermore, the thermal stabilities of the modified lysozyme preparations have been investigated under denaturing conditions in 3 *M* guanidinium hydrochloride at pH 5.5. It was found that one bis(bromoacetamide) reagent $[BrCH_2CONH]_2(CH_2)_4$ stabilized the lysozyme derivative to a high degree whereas the cross-linkers containing shorter or longer spacers enhanced the stability of lysozyme to a weaker degree. Therefore, it was concluded that maximum stabilization can be achieved only when the proper length of a cross-link is introduced into a protein. Thus, intramolecular covalent bridging may be useful in stabilization of structural characteristics of proteins and maintenance of their biological activity if denaturing solvents are applied.

Chemical cross-linking and chromatographic separations have also been used to investigate the conformation of two members of the integrin family, rat

$\alpha_1\beta_1$ and human $\alpha_{IIb}\beta_3$ integrin [35,66,98]. As mentioned in Section 4.2, integrins are heterooligomeric, non covalent assembled transmembrane glycoproteins. They mediate cell-matrix and cell–cell adhesion thereby connecting the extracellular environment with the intracellular compartment [99]. One special feature of this class of membrane receptors concerns their ability to adopt variable conformations of the extracellular domain, which result in modulations of their ligand binding activity and regulations of intracellular effector protein binding. If there are variant conformational states, the conformers carry different cross-linker accessible functional groups of their surfaces. Thus, cross-linking should produce individual molecular bridges while freezing three-dimensional conformations. Now, denaturing conditions as used in SDS-PAGE cannot destroy the conformations of the molecules of interest. If the cross-linked conformers possess variant hydrodynamic volumes, they should be separated by SDS-PAGE resulting in bands with distinct relative molecular masses.

Investigation of integrin species were made as follows (see Fig. 5) [35,66]: Membrane proteins were solubilized by detergent from enriched plasma membrane fractions and treated with DSS/DSP. $\alpha_1\beta_1$ integrin was immunopurified using anti α_1 subunit Sepharose (immobilized monoclonal antibody), whereas $\alpha_{IIb}\beta_3$ integrin was enriched by IEC on DEAE-Sepharose. Surprisingly, chemical modification by DSS/DSP did not change the net charge of $\alpha_{IIb}\beta_3$ integrin; binding/elution in IEC was not diminished. SDS-PAGE and immunoblotting showed that cross-linking produced high molecular mass protein complexes of both integrins ($\alpha_1\beta_1$ integrin: M_r $2.40/2.90\times10^5$; $\alpha_{IIb}\beta_3$ integrin: M_r $2.0/2.20/2.40\times10^5$). To investigate the nature of the high molecular mass species, the single aggregates were purified by preparative SDS-PAGE and analysed again after reductive cleavage with dithiothreitol. However, the cleavage analysis of these complexes revealed that the cross-linked aggregates contained only single integrin subunits in roughly stoichiometric ratio. Other proteins associated with the integrins were not detected. Thus, the data indicate that the $2.40/2.90\times10^5$ rel. mol. mass units species of $\alpha_1\beta_1$ integrin and the $2.0/2.20/2.40\times10^5$ rel. mol. mass units species of $\alpha_{IIb}\beta_3$ integrin represent heterodimers cross-linked in different conformations, thereby producing molecules with different electrophoretic mobilities in SDS-PAGE. It is still unclear how the different conformations are formed and whether they determine the biological activity of the investigated integrins. Peptide mapping of purified cross-linked species is needed to clarify the molecular basis of integrin cross-linking.

In conclusion, intramolecular cross-linking provides an experimental tool for the detection, isolation and subsequent structural characterization of near neighboured domains within a protein. In addition, molecular bridging also offers the analytical approach to study conformations of a given protein.

5. Conclusion

The maintenance of intra- and intermolecular protein interactions is a prerequisite for analysis of supramolecular protein aggregates under native conditions. Chemical cross-linking represents a simple experimental concept that provides this requirement. Because it freezes native molecular associations by covalent bridging, the original composition of protein aggregates is preserved even under denaturing conditions, as usually utilized for protein purification and analysis. Thus, the individual spatial relations of a given protein or protein complex may be directly analyzed. Because cross-linking generates molecular aggregates with higher M_r as compared to the M_r of non cross-linked species, its progress can be easily monitored by SDS-PAGE/immunoblotting.

Since the introduction of reagents with defined chemical characteristics and molecular sizes, cross-linking has been applied to investigation of both inter- and intramolecular associations as well as three-dimensional configurations of proteins. Among the reagents used are derivatives of NHS- and imidoesters that react specifically with primary amino groups, derivatives of maleimides with specificity towards sulfhydryl groups and variant carbodiimides that target carboxyl groups. The variety of cross-linkers is further extended by reagents with different reactive groups and by photoactive reagents which remain chemically inactive until they are exposed to ultraviolet illumination. They have been especially applied in studies of receptor–ligand

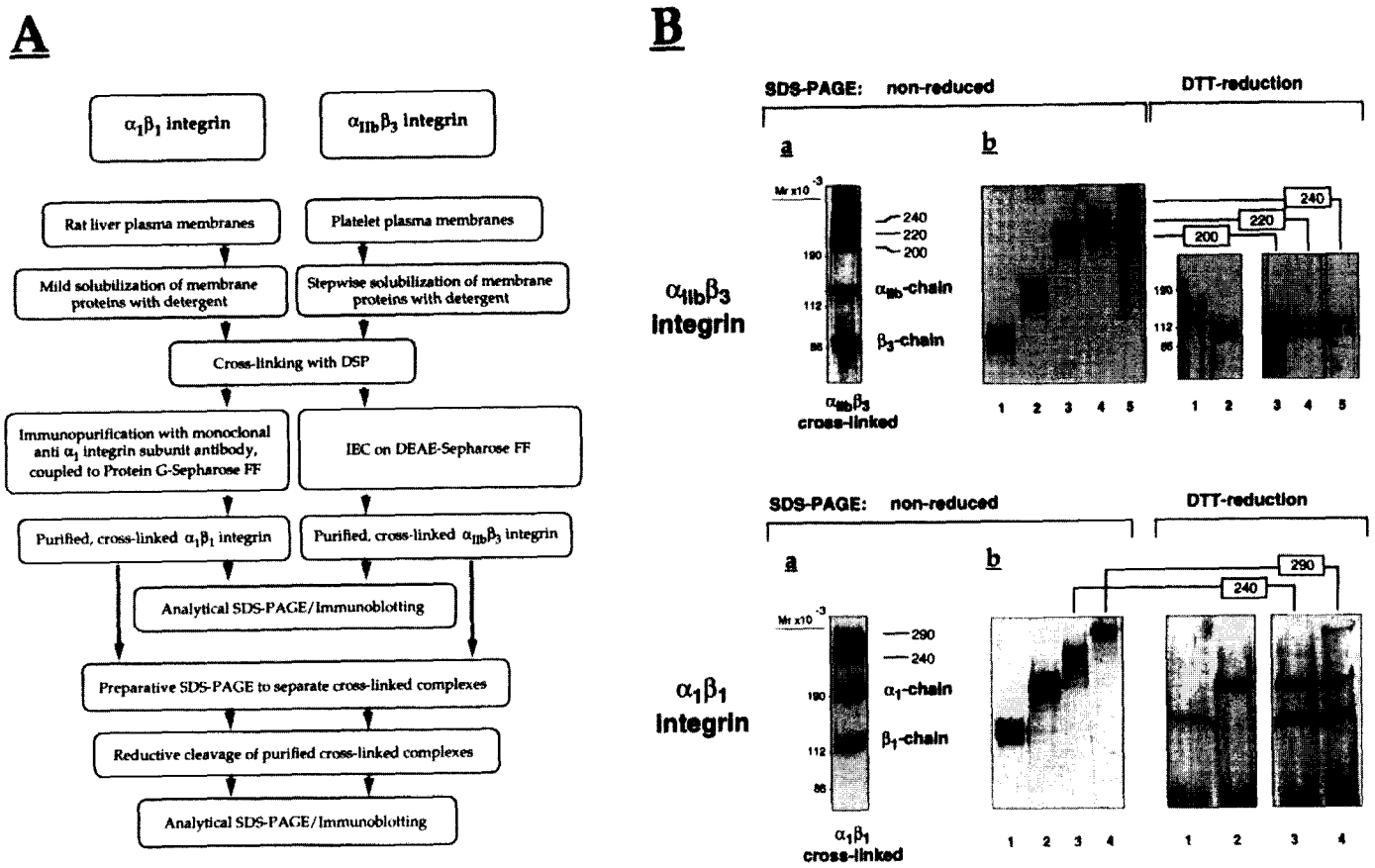


Fig. 5. Cross-linking analysis of integrins. (A) Scheme of experimental steps. (B) SDS-PAGE and immunoblot analysis of cross-linked purified $\alpha_1\beta_1$ and $\alpha_{IIb}\beta_3$ integrin. Samples of solubilized membrane proteins from rat hepatocytes and human platelets were treated with DSP. Fractions containing purified $\alpha_1\beta_1$ and $\alpha_{IIb}\beta_3$ integrin ($\alpha_1\beta_1$ cross-linked, $\alpha_{IIb}\beta_3$ cross-linked) were subjected to preparative SDS-PAGE for separation of high M_r complexes and non cross-linked, single integrin chains followed by analytic electrophoretic separation of each of its components either under non-reducing or reducing (DTT-reduction) conditions as indicated. Proteins were visualized by silver staining or immunoblotting with polyclonal antibodies against the corresponding integrin subunits. As seen in the figure, all cross-linked aggregates are composed of the corresponding integrin subunits in roughly stoichiometric ratios. No cross-linked, integrin-associated proteins were found (modified from Löster et al. [35,66], reprinted with kind permission from Refs. [35,66].

interactions. A further significant advantage of some cross-linkers is given by their cleavability which can be achieved under mild reducing conditions. However, every cross-linking application needs an individual protocol that has to be improved at the beginning of investigations.

As outlined above, chromatographic separations may be combined with cross-linking in different ways. A frequently used approach takes cross-linking to confirm the existence of a given protein in complexes which were first detected by SEC, whereas the identification of unknown binding partners of known proteins starts with cross-linking prior chromatographic purifications. However, both methodologies do not provide information about the molecular basis of protein assembly. The detailed molecular analysis can be only achieved if the purified cross-linked species are studied by protein-chemical methods like peptide mapping, N-terminal sequencing and mass spectrometry thereby using IEC and RPC to isolate single fragments. Because cross-linking occurs at distinct locations within a protein, its localization provides direct information on neighboured components/domains within a protein aggregate. This seems to be one of the most interesting features of this methodology making it an universal tool for investigations of protein associations.

6. List of abbreviations

AC	Affinity chromatography
IAC	Immunoaffinity chromatography
IEC	Ion-exchange chromatography
M_r	Relative molecular mass
RPC	Reversed-phase chromatography
SEC	Size exclusion chromatography

Nomenclature of cross-linkers (* photoactivable reagents)

ANB-NOS*	*N*-5-azido-2-nitrobenzoylsuccinimide
BASED	Bis-[β-(4-azidosalicylamido)ethyl] disulfide
BBA	Bis(bromoacetamide) derivatives
BMH	Bismaleimidohexane
BSS	Bis(sulfosuccinimidyl)suberate
BSOCOES	Bis[2-(succinimidooxycarbonyloxy) ethyl]sulphone
DB	Diepoxybutane
DCC	Dihexylcarbodiimide
DFDNB	1,5-difluoro-2,4-dinitrobenzene
DMA	Dimethyl adipimidate
DMS	Dimethyl suberimidate
DTBP	Dimethyl 3,3′-dithiobispropionimidate
DSP	Dithiobis(succinimidylpropionate)
DSS	Disuccinimidylsuberate
DST	Disuccinimidyl tartarate
DTSSP	Dithiobis(sulfosuccinimidylpropionate)
DTNB	5,5′-dithiobis(2-nitrobenzoic acid), Ellman's reagent
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
EGS	Ethyleneglycol bis(succinimidylsuccinate)
FNPS	4,4′-Difluoro-3,3′-dinitrodiphenyl sulfone
GA	Glutaraldehyde
HSAB*	*N*-hydroxysuccinimidyl-4-azidobenzoate
IT	2-Iminothiolane
MBS	*m*-Maleimidobenzoyl-*N*-hydroxysuccinimide ester
NFPLP	2-Nor-2-formylpyridoxal
PPDM	Para-phenyl dimaleimide
SADP*	*N*-succinimidyl[4-azidophenyl]1,3′-dithio)propionate
SANPAH*	*N*-succinimidyl-6-(4-azido-2′-nitrophenylamino)hexanoate
SMCC	Succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate
SMPB	Succinimidyl 4-(*p*-maleimidophenyl) butyrate
SMPB	Succinimidyl 4-(*p*-maleimidophenyl) butyrate
SPDP	*N*-Succinimidyl 3-(2-pyridyldithio) propionate
sulfo-SADP*	Sulfosuccinimidyl(4-azidophenyldithio)propionate
SSASDP*	Sulfosuccinimidyl 2- (4-azidosalicylamido)ethyl-1,3-dithiopropionate

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