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Review

Chemical cross-linking and protein–protein interactions—a review with illustrative protocols

Ronald Kluger*, Amer Alagic

Davenport Chemical Research Laboratory, Department of Chemistry, University of Toronto, Toronto, Ont., Canada M5S 3H6

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Abstract

The general term "protein–protein" interactions refers to the effects of proteins upon each other. The interactions can arise from co-existence in organized structural arrangements or in transient encounters. The latter are difficult to detect and define. Introduction of specific, stable chemical linkages can establish permanent relationships between what would normally be transiently associated species. The review covers the types and purposes of various linkers, including the comparative advantages of various approaches. The emphasis is on practical applications and thus includes methodology in the form of practical protocols for introducing the linkages and interpreting the outcomes.

Keywords: Protein–protein interactions; Chemical cross-linking; Protocols; HPLC analysis; Structure; Peptide maps

1. Introduction

1.1. Defining the area

Protein–protein interactions occur where proteins or portions of proteins approach each other at close range. To be significant, the interaction should be

* Corresponding author. Fax: +1 416 978 3582.
E-mail address: rkluger@chem.utoronto.ca (R. Kluger).

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structurally defined—not a random collision. Specific interactions are associated with catalysis of reactions, control of a protein's activity, stability of proteins within complex species, and control of assembly of complex structures. Identifying the sites of the interactions provides a basis for forming an integrated picture of the functional proteins. Where high-resolution methods for structural analysis are applicable, the stable interactions within a protein can be determined by inspection of the solved structure. Theoretical modelling reveals further interactions with the solved structure as a starting point. For proteins that are an assembly of subunits, information about structural details may also be accessible by physical methods. Where subunits undergo motions relative to one another in response to effectors, multiple structures must be solved. In multi-protein complexes relatively large-scale motions can occur during processing of complex substrates so protein–protein interactions are inherently dynamic. Solving such structures in detail is a challenge that has had some notable successes as exemplified by the elucidation of the structure of the photosynthetic reaction center [1,2].

An alternative approach is to build up a set of structurally defined interactions on a one-to-one basis with a permanent record. We know a fish has approached the hook at the end of a fishing line if we find the bait is missing. If the fish becomes attached to the hook, the point of interaction becomes permanently defined. If we "fish" for protein–protein interactions we can use chemical "baits" and "hooks" to produce permanent records of interactions. Chemicals that modify proteins can be designed to have an affinity for specific sites or types of sites. By connecting two or more protein-directed reaction sites into one chemical, we provide the permanent mark that indicates the site of a protein interaction as well as a cross-link that will hold it together. Analysis of the site of cross-linking reveals where spatially defined interactions occur. In this review we illustrate the methods and principles of chemical cross-linking in reporting and stabilizing protein–protein interactions, as well as examples of materials that can be used for this approach.

1.2. Classifying cross-links in protein–protein interactions

Since the term "protein–protein interactions" can represent many types of interactions, for the purposes of this article it is helpful to consider the possibilities in a systematic way, based on the effects of cross-linking. In a single polypeptide chain, a cross-link can be created within that chain, denoting regions that approach within the span of the linker. This can be written as an (A–A′). If a protein contains multiple subunits, a link is indicated: (A–B). If a single-chain protein is part of a multi-protein complex, then a link between different proteins in the complex would be (A)–(M). For multi-subunit proteins, we could have (AB)–(MN). If these had inter-subunit cross-links, they would be (A–B)–(M–N). If the second component of the link is part of a remote or unrelated multi-protein complex, then we use letters near the end of the alphabet: (AB)–(XY). This classification of protein–protein interactions can help clarify the nature of the stated result since the term "protein–protein" is inclusive and non-specific.

1.2.1. The steps in cross-linking and analysis

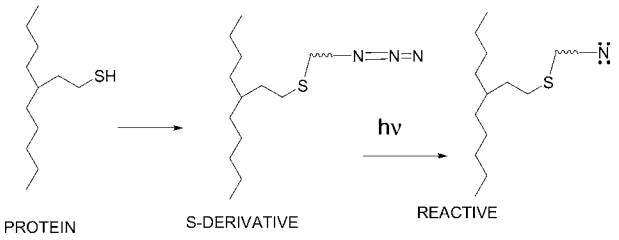
Selection of a reagent is critical. If the target sites are known, then the reagent is selected for its specificity. This limits the number of products, maximizes the yield, and facilitates identification of the sites of reactions. If the same functional group is targeted on both entities, a homofunctional reagent [3] may achieve the desired result. In other cases, heterofunctional reagents [3] can differentiate regions or specific proteins. A common problem in using the reagents is the competition with other processes, such as hydrolysis or internal reaction (within a protein) where external reaction (between subunits or proteins) is desired. Control can be achieved by selection of a connecting chain within the reagent that is directed away from the original site [4]. Selective activation of the linking group after the initial step, for example, with subsequent photoactivation (see next section), is an interesting alternative [5,6]. Once the reagent has been chosen, the process begins with reaction of the first site in a protein. This is followed by reaction of the second site to be connected with the reagent. The products must then be identified and the loction of the link determined. It is usually necessary to separate products to continue identification (chromatography and isoelectric focusing are commonly used). The different pools are separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), immunoprecipitation, or western blot analysis. Sufficient data for structural analysis by tryptic or similar digestive peptide mapping can then be obtained. However, detection and isolation of the modified portion of the proteins is often difficult, even with radioisotopic labelling of the reagent, so affinity methods are being developed. Mass spectral determination of the identity of products, especially with the aid of a database, greatly facilitates the process [7–9].

2. Interesting approaches

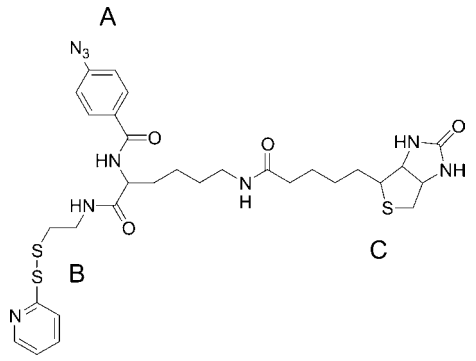
2.1. Strategies for stepwise reaction

In principle, any bifunctional reagent can connect two structures. There will be no control over which end reacts with which piece, unless the reagent has specific affinity or the targeted residue in one partner has no counterpart in the other. A recently developed alternative is to have the second group be inactive until after the first reaction is complete [6]. Several applications have been reported in which the second reaction is photo-activated [5,10]. These "photoaffinity reagents" [11–16] become reactive upon photolysis, generating a nitrene derivative from an unreactive azide precursor. The result is an extremely reactive and unselective species, which will create a link to any bond that can be part of the insertion process. By linking the reagent initially to a specific residue (usually a thiol that is introduced by site-directed mutagenesis) in the separated structure, photoaffinty cross-linking can then reveal interactions, such as (AB)–(MN). A radioactive group can easily be incorporated to assist in locating the modification. For example, von Hippel introduced cysteine at a specific site in a protein of a bacteriophage DNA polymerase assembly [6]. In order to determine the relative

position in the complex of the modified protein and the other components, he utilized a sulfhydryl reagent that reacts with the introduced cysteine. The reagent also has a nitrene precursor that is activated by light after the reagent is attached to the protein [6]. von Hippel's reagent is the maleimide (TFPAM-3), which is a propionylmaleimide derivative that reacts with cysteine. The residual aryl azide of TFPAM-3 is available for photo-cross-linking of the protein after modification. Photo-cross-linking produces a permanent connection to neighbouring entities. The resulting structure was analysed by SDS–PAGE and western blotting. The connected subunits were identified with polycolonal antibodies. These results and others reported in the paper led to a spatial model of the complex and the changes induced by reaction with ATP, as well as a mechanistic model for DNA synthesis in this system.



Benkovic used a related reagent for photo-cross-linking to identify (A)–(MN) protein–protein interactions that utilizes the affinity of avidin for biotin to allow easier detection and isolation of the cross-linked product [10]. The commercially available trifunctional reagent sulfo-SBED is a modified form of lysine. The α-amino group is connected as an amide to the photolabile *p*-azido benzoyl group (**A**). In sulfo-SBED the lysine carboxyl is an amide of a 2-thioethylamine disulfide derivative containing an active ester that will react non-specifically with lysines. Benkovic exchanged the disulfide with the thiopyridine leaving group (**B**) so that the reagent will react with a specific cysteine. The ε-amino group of the lysine is acylated by the side chain biotin (**C**).



The denatured monomeric proteins from the gel that contain biotin can be detected by their chemiluminescence or extracted by their affinity. The permanent nature of the insertion into the protein chain allowed sequencing to be used to determine the sites that are modified, which is facilitated by the attachment of biotin for recognition. Benkovic predicts a bright future for cross-linking in detecting protein–protein interactions as a tool to: "... facilitate the investigation of important biological systems that require the formation of multiprotein complexes, for example, DNA replication, transcription, recombination, DNA repair, signal transduction, and molecular recognition. In the investigation of highly complex systems, the use of affinity probes becomes increasingly essential." [10].

2.2. Cross-linking transient structures to detect product channelling

Bernhard proposed that transient interactions between proteins allow transfer of the product of one protein for processing by another ("channelling"), avoiding problems of dispersion and diffusion [17–19]. The process is implied by very large rates of reaction in a two-enzyme process. Stubbe examined the case of phosphoribosylpyrophosphate amidotransferase (PRPP-AT) and glycinamide ribonucleotide synthetase (GAR-syn), in which phosphoribosylamine is a product of PRPP-AT and a substrate of GAR-syn [20]. The reagents MCCH (a hetero-bifunctional reagent containing maleimide and an NHS ester) and SMPB [succinmidyl 4-(*p*-maleimidophenyl)butyrate] cross-link PRPP-AT to a second molecule of the same enzyme (A)–(A), independent of the presence of substrates or GAR-syn. There is no capture of the two proteins together (A)–(M). Since the cross-linking reaction must be slow compared to substrate transfer, the result shows that an altered approach will be necessary to achieve this type of connection.

3. Practical considerations

The existence of many commercially produced reagents intended for cross-linking provides a good initial approach to consider possible strategies. Reviews [3,21,22], catalogues [23], and a monograph [24] help in achieving a rapid familiarity with the existing approaches. Further design and synthesis, often by modifying commercial materials, can give new candidates. With feedback from the results of the first sets of experiments, the reagent can be improved or other approaches attempted. In 1977, Uy and Wold summarized the state of the field [25]: "A number of bifunctional protein reagents were developed; initially they were applied mostly to induce increased strength to structural proteins and stability to globular ones, or as 'yardsticks' to measure interresidue distances in cross-linked protein derivatives, but their use was rapidly expanded to exploration of the spatial arrangement of subunits in oligomeric proteins and of the organization of protein molecules in more complex structures such as ribosomes and membranes." The idea of analyzing protein–protein interactions by cross-linking was already established! Uy and Wold also classified types of cross-linkers by their functional groups, defining the standard categories

that are still the only widely used system. A 1997 review provides insights into the practical issues of creating a cross-link by designing new reagents [26].

3.1. Effects of the cross-link

A cross-link cannot be introduced without altering the proteins that are being analysed, making the end result subject to interpretation. The reacted material may be functionally similar to the native or it may not be. If it is not, then the basis for the alteration introduces more questions. For example, cross-links in hemoglobin can alter its cooperativity and its affinity for oxygen. The variation in oxygen affinity can depend on the location and size of the cross-link.[27,28] A cross-link introduces a permanent relationship where there may be a need for frequent separation in functioning. The reagent itself may induce electrostatic or hydrophobic attraction, causing sites to react with each other that normally are not in proximity, perhaps giving a false or deceptive report of an interaction. Results reported in terms of altered function as well as location require considerable interpretation. One approach to isolating the effect of the cross-link from that of the chemical modification is to produce a link that can be cleaved after it is formed [3]. For example, if there is a disulfide within the cross-link, it can be reduced and blocked to prevent recombination. The resulting product can be compared to the altered protein with the cross-link intact, as well as with the native material. This potentially informative approach severely limits the reagents that can be employed and is not necessary if the location of interactions is the prime consideration. Another approach that is more general could involve a control experiment using a monofunctional analogue of the cross-linking reagent. The protein is modified under the conditions of the cross-linking reaction with the resulting material compared to the native and cross-linked proteins.

3.2. Selecting a reagent

In order to use cross-linking as an effective tool for studying protein–protein interactions, the choice of reagent should be based on the specific properties of the targets. Information from chemical modification studies is especially useful [29]. For example, if different affinity reagents react efficiently with each of the partners, we would select or design a cross-linking reagent that combines these features with an appropriate spacer. Reagents that resist spontaneous processes such as hydrolysis are best suited for the purpose. Still, the more common approach has been to try known, readily available reagents. This can give a much broader set of products in lower yield than would come from a selective reagent. If the reagent's selectivity is defined by specificity for a functional group (chemoselectivity), then because many amino acid side chains occur at many sites in a protein, such reagents will over-modify the protein. With the possibility of multiple reaction sites, a heterogeneous mixture is the most likely outcome of an initial experiment that produces any type of cross-link. Even where stoichiometric amounts of reagent are used, we can expect to find multiple modifications of a single protein chain. With residues on several side chains showing equal reactivity toward a reagent, it is equally likely that two

molecules of reagent will react at two sites on one protein molecule as well as on one site on each of two protein molecules. The use of introduced cysteines and their reaction with sulfhydryl reagents is a modern approach to solving the problem but it requires expression and mutation.

3.3. Selectivity in cross-linking by design

How can chemically introduced cross-links be as useful as possible for studies of protein–protein interactions? In the ideal case, the connected system continues to be active, confirming that a viable form of the material has been identified. One might design selectivity by creating reagents that are analogues of an enzyme's substrate. In the case of channelling, the reagent could be analogous to the substrates of the two enzymes at each end. However, these will necessarily inactivate an enzyme or enzymes whose interactions are being studied. An alternative is to utilize an analogue based on an allosteric effector (where these exist).

3.3.1. Selective reagents for cross-linking

The use of unselective reagents will give little information about specific interactions, so the most useful study will take advantage of specific parts of the proteins' structure. Reagents should target particular areas of the protein, if not a specific site, as well as a type of functional group within that area. A reagent that recognizes charge density, solvent exposure, or hydrophobicity might accomplish this. A product of the modification reaction should discourage further reaction in its vicinity. The structure linking the reacting groups should be well defined in terms of conformation, not an extended, flexible chain. In cross-linked hemoglobins, reagents produce selectivity for a single site on each subunit, permitting cross-linking to report systematically on subunit interactions [30].

4. Strategic approaches

4.1. Multifunctional reagents for improved yield and selectivity

Some reagents with more than two reaction sites have inherent advantages. If only two sites on the reagent react, the other sites can guide the reagent to a particular region of the protein, provide a handle for recognition, or permit subsequent reaction. A trifunctional reagent can often give a higher yield of a desired product than a similar bifunctional reagent [31–33]. If a bifunctional reagent reacts with a desired site and an undesired site, it becomes impossible for the reagent to connect the intended sites. A trifunctional reagent in which the reacting groups are similarly placed can continue to react. The trifunctional reagent trimesoyl tris(methyl phosphate) was used to link lysine-82 of one beta subunit of hemoglobin with the alpha amino group of the N-terminal valine of the other subunit. Formation of a link between lysine-82 on both beta subunits competes, as does hydrolysis of the reagent. With the bifunctional reagent, isophthalyl bis(methyl phosphate), a mixture of products results with

only a small amount connecting beta subunits between lysine-82 and valine-1. The trifunctional reagent introduces the 1–82′ link with much greater efficiency.

4.2. Cross-linking followed by cross-linking

If a large excess of the cross-linking reagent is needed for reaction to be achieved, it is likely to produce a mixture of products that will be difficult to analyse. On the other hand, after a specific cross-link is established with a selective reagent, a second link can be created with a reagent with different specificity. Thus, in a multi-protein complex, links between different pairs of protein could be introduced in a series of reactions with different reagents.

4.3. Importance of the link's structure

The ends of the link are defined by the functional groups that react. The rest of the link is defined by the structure of the reagent. If the link is flexible it is not safe to assume that the chain is fully extended. In the simplest and easiest to interpret case, the bridge is a linear connection of defined length between known positions in side chains. In studies of protein–protein interactions forming the connection is central to the study and the nature of the link has ordinarily not been evaluated. Based on the results we have seen in assessing the effects of links between subunits in hemoglobin, this aspect should receive more attention. A bridge having a structure that maintains its span provides a defined distance between the connected entities and can provide feedback in optimising yield and stability.

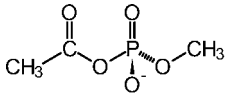
4.4. Commercial catalogues

Two commercial catalogues specialize in reagents for cross-linking that apply to issues in this chapter. Pierce Chemical Company of (Rockford, IL) includes a detailed section on cross-linking with their products [34]. The section reflects the company's approach to the product line and their ideas about how the materials might be used. There is a general introduction, tables of reagents with their uses, discussions of tactics, a glossary of terms related to cross-linking, and a bibliography. They use the Uy–Wold classifications and refer to the chemical structure between the functional groups of the reagent as a "spacer arm." The chains featured in Pierce's catalogue mostly are modified aliphatic hydrocarbons. Molecular Probes, Inc. (4849 Pitchford Ave., Eugene, OR 97402-9165) produces materials that have novel properties such as photoreactivity. The nature of the linking structure is coincidental. They offer an on-line "handbook" (http://www.probes.com/handbook/) with a section on photoreactive reagents and applications in protein–protein interactions, as well as other sections on cross-linkers in general. A discussion of von Hippel's reagent mentioned earlier is as follows: "The polyfluorinated maleimide azide TFPAM-3 (*N*-(4-azido-2,3,5,6-tetrafluorobenzyl)-3-maleimidylpropionamide) is perhaps the most efficient reagent for photo-cross-linking thiols to adjacent residues. TFPAM-3 has been used to identify the interacting subunits of F_1F_0-ATPase

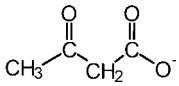
involved in conformational changes associated with ATP synthesis and hydrolysis and for structural analyses of bacteriophage T4 DNA polymerase holoenzyme. Another polyfluorinated maleimide, TFPAM-SS1 (*N*-(2-((2-(((4-azido-2,3,5, 6-tetrafluoro)benzoyl)amino) ethyl)dithio)ethyl)maleimide) undergoes thiol reaction and photolytic nitrene insertion to form reversible crosslinks that can be cleaved by disulfide reduction. The internal disulfide in the crosslink can be reduced ... to yield free thiols, which can then be derivatized with fluorescent or radioisotopic labeling reagents to allow identification of the crosslinked products.'' They are advocates of this methodology and provide some unique materials.

5. Affinity cross-linking

Bifunctional analogues of affinity labels may serve as cross-linking reagents for proteins where location rather than activity is of interest. Bacterial D-3-hydroxybutyrate dehydrogenase is a highly specific dimeric enzyme. Methyl acetyl phosphate (MAP), an analogue of the substrate, acetoacetate, irreversibly inactivates the enzyme by acetylation of an essential lysine at the active site [35]. There are two active sites per dimer.



Methyl Acetyl Phosphate

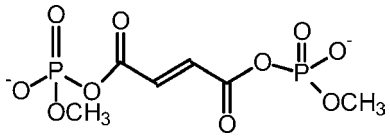


Acetoacetate

Fumaroyl bis(methyl phosphate) is a bifunctional analogue of MAP. One equivalent of this material cross-links the protein across its active sites.

5.1. A sample protocol for affinity cross-linking

Fumaroyl(bis methyl) phosphate cross-linking of D-3-hydroxybutyrate dehydrogenase [35]



The cross-linking reagent is not commercially available but it is readily prepared.

Materials

Sodium iodide
Tetrahydrofuran
Acetone
D-3-hydroxybutyrate dehydrogenase

Methods

Synthesis of the cross-linking reagent:

1. Mix fumaroyl chloride with two equivalents of sodium dimethyl phosphate suspended in tetrahydrofuran to give fumaryl bis(dimethyl phosphate). The reaction proceeds most efficiently if the vessel is ice-cooled. The reaction is completed in less than 30 min and should not be left longer.
2. Treat the tetraester with two equivalents of sodium iodide in acetone to obtain the cross-linking reagent as a precipitate, which is collected by filtration and washed with cold, dry acetone.

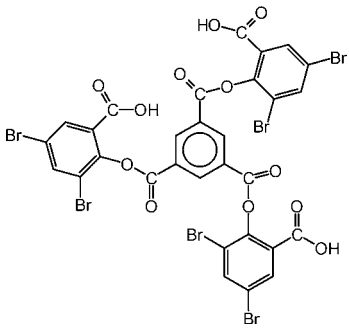
Cross-linking with fumaroyl bis(methyl phosphate)

1. Add the solution of enzyme (0.35 units/mL) to 1–2 mL of a solution of fumaroyl bis(methyl phosphate) (concentration between 2.5 and 20.0 mM).
2. Incubate the reaction mixture at 25.0 °C at pH 7.0 and withdraw aliquots for assay of activity until at least 99% of original activity is lost. After 30 min of incubation, most of the enzyme activity is lost.
3. SDS–PAGE gel electrophoresis (5 and 7.5% in acrylamide monomer with 0.3% methylene bis-acrylamide) with molecular weight standards before and after treatment with reagent indicates formation of a band with twice the weight of the subunits in the original enzyme. The original bands are no longer present.

5.2. Steric and electrostatic selectivity

The bifunctional anionic reagent, fumaryl bis(3,5-dibromosalicylate) cross-links the subunits of the hemoglobin tetramer between α lysines-99 as well as between β lysines-82, regions of high positive charge character. The reagent can be diverted exclusively to the α lysines-99, by conducting the reaction in the presence of large amounts of polyanions (most commonly inositol hexaphosphate), which bind preferentially to the reactive amino groups of the beta subunits. The protein remains functional with the reagent after cross-linking. The high yield of a single cross-linked protein is dramatic support for the electrostatic-directed approach. Since that result is specific for hemoglobin, the protocol will not be given here. Variation of the substituents on the aromatic rings of the leaving group showed that the dibromo reagent is the most selective, probably by a combination of steric and electrostatic effects [31].

In order to enhance the selectivity of the reagent and to provide for a third reaction site, we prepared material with three reaction sites, trimesoyl tris(3,5-dibromosalicylate) [32,36]. This is not accessible by direct reaction from the components, requiring protection of the carboxyl groups.



Protocol: cross-linking hemoglobin with trimesoyl tris(3,5-dibromosalicylate) [36]

Materials

0.1 M sodium borate buffer, pH 9.0
0.1 M (3-[*N*-morpholino]propanesulfonic acid) (Mops) buffer, pH 7.2
Dioxane, "CAUTION" (highly flammable, harmful)
TTDS
CO, "CAUTION" (extremely flammable, very toxic)
Oxygen
Hemoglobin

Methods

1. Twenty millilitres 0.1 M borate, pH 9.0 (from boric acid and sodium hydroxide) is combined with 1.44 g hemoglobin (CO form) in 18 mL of water .
2. Bubble oxygen through the solution of hemoglobin on ice to remove the CO for 2 h.
3. Deoxygenate by passing nitrogen through the solution at 37 °C for 2 h.
4. Combine 2.5 mL dioxane, 2.5 mL 0.1 M, pH 9.0, borate buffer, and 2.0 mL water. Add 37 mg TTDS.
5. Add the TTDS solution to the hemoglobin solution and mix gently. (Minimize exposure of deoxyhemoglobin to air to prevent formation of methemoglobin.) After 2 h cool the reaction in ice, convert to COHb by passing CO through the solution for 10 min with mixing.
6. Pass the solution of carbonmonoxyhemoglobin cross-linked with TTDS through a Sephadex G-25 column equilibrated with 0.1 M Mops buffer, to remove unreacted TTDS.

5.2.1. Analysis

If cross-linking has occurred between chains or dissociable subunits, an analytical method sensitive to molecular size is essential. PAGE and size exclusion chromatography are useful if there is information from similarly sized structures available. Modern mass spectral methods have greatly improved the ease and accuracy of weight analysis and identification. After reaction of proteins with a reagent expected to produce a cross-link, it is unlikely that a single modified product will result. Therefore, separation of the modified proteins is necessary, or a method of analysis can be chosen that deals with individual components. If a method has been found to separate the components, the extent and nature of the modifications should be known and the individual species can be studied. Characterization of physical properties and detailed structure are important goals. The analytical procedures summarized below follow the procedures outlined by Jones for hemoglobin [37]. Specific protocols are not given since these depend on the protein and reagents used.

5.2.2. Estimating heterogeneity by ion exchange HPLC

Modification of the protein with most cross-linking reagents is likely to change the net charge at the modified residues. Therefore, methods that are sensitive to electrostatic differences are most suitable for initial attempts to determine the number of components in the sample. Analytical HPLC using both anion exchange and cation exchange columns gives more possibilities for separations. Analysis of structural modifications is best interpreted with confidence if the components can be separated. Ion exchange HPLC procedures can be scaled to produce materials found in analytical columns. Alternatively, column chromatography with a support such as DEAE-Sephacel of CM-Sephadex can give the desired separation of the modified proteins.

5.2.3. Protein analysis and separation

Reversed phase C4 HPLC columns are useful for procedures involving subunits of multimeric proteins. These can be scaled to preparative levels. SDS–PAGE analysis is useful in order to determine if the individual subunits are cross-linked. More detailed information can be obtained by combining HPLC with mass spectrometry, which gives the exact molecular weight of the eluting species and characteristic decomposition patterns. If the primary structure of a protein is known then peptides from digests can be compared before and after modification. For example, conversion of amino groups to amides will block hydrolysis reactions normally catalysed by trypsin. Some tryptic peptides will not be present and new ones should appear on chromatographic separation. The peptide can be detected spectrophotometrically if there is a chromophore or by mass spectrometry, even if there is no chromophore. If the peptides cannot be identified from their elution patterns, amino acid analysis combined with sequence determination is necessary.

6. Systematic examples of analysing protein–protein interactions by cross-linking

6.1. Different types of linkages

6.1.1. Type (A)–(A′): cross-linking within the same polypeptide chain

Systematic variation of cross-link structure has rarely been used. However, an interesting example has been reported in the case of myosin. This protein undergoes a conformational change upon binding nucleotides and actin. The flexible structural elements of myosin are involved in coupling conformational change to movement. The conformational dynamics of the movement were investigated using cross-linking reagents of different lengths [38–40]. Burke and co-workers used three cross-linking reagents: 2,4-dinitro-1,5-difluorobenzene (F_2DNB), 4,4′-difluoro-3,3′-dinitrophenyl sulfone (F_2DPS), and *p*-*N*,*N*′-phenylenedimaleimide (pPDM) with lengths of 5, 7–10, and 12 Å, respectively. The reagents targeted sulfhydryl residues SH_1(Cys707) and SH_2(Cys697). In the absence of nucleotide, only the pPDM, the longest cross-linking reagent (12 Å), was able to cross-link SH_1 and SH_2. However, in the presence of nucleotide, F_2DPS, the shorter cross-linker, was able to form a cross-link as well. Therefore, they were able to demonstrate that the distance between two essential cysteine residues is sensitive to nucleotide binding.

Protocol: cross-linking myosin [38,39]

Materials

Myosin (myosin in 0.5 M KCl, 0.01 M EDTA can be stored in 50% glycerol at −20 °C. Before use, dialyse against 0.5 M KCl, 0.5 M Tris, pH 7.9, dilute to 3–6 mg/mL, and dialyse against 0.3 M KCl, 0.025 M Tris, pH 7.9)
Stock solutions of FDNB, F_2DPS, and pPDM in acetone
Conjugation buffer: 0.3 M KCl, 0.025 M Tris, pH 7.9
Stop buffer for pPDM: 0.5 M KCl, 0.05 M Tris, 0.002 M DTT, pH 7.9

Methods

1. Prepare stock solution of myosin (5 mg/mL) in 0.3 M KCl, 0.025 M Tris, pH 7.9.
2. Dissolve the required amount of the reagent in acetone (for cross-linking reaction with F_2DPS and pPDM use 1 mol of bifunctional reagent per mol of myosin head; for reaction with FDNB use fourfold excess of the reagent).
3. Mix 0.5 mL of myosin stock solution and appropriate amount of cross-linking reagent for 30–35 min at 5 °C.
4. Terminate reaction by precipitation with 15 volumes of cold water followed by centrifugation. Precipitated protein is dissolved in 0.5 M KCl (pH 7.5) and precipitated twice with cold water. 4a. Cross-linking reaction of myosin with pPDM is terminated by dilution of protein in stop buffer.

6.2. (A)–(B): cross-linking between two polypeptide chains

Chemical cross-linking was used to investigate subunit interactions in luteinizing hormone. The protein is a αβ dimer and its function depends on interactions between the hormone and its receptor, as well as on interactions between the α and β subunits. van Dijk and Ward studied α−β subunit interactions using EDC and found that the lysine residue that is cross-linked by the reagent is at the interface between the subunits [41].

Protocol: cross-linking of porcine luteinizing hormone with EDC [41]

Materials

Porcine luteinizing hormone
EDC
0.001 N HCl "CAUTION" (corrosive)

Methods

1. Dissolve hormone in deionized water to a concentration of 0.2 mg/mL.
2. Adjust pH to 4.75 with HCl.
3. Dissolve EDC in deionized water and add to the hormone solution to the final concentration of 0.02 M.
4. Mix at room temperature for 1.5 h, keeping the pH constant.
5. Dialyse reaction mixture against 0.001 N HCl, and lyophilize.

6.3. Cross-linking between two different proteins within the same complex (AB)–(MN)

The analysis of protein–protein interactions between proteins within a complex is usually more challenging than creating cross-links with one protein. The number of interactions within such a complex is large and conjugates with high molecular mass may form that cannot be readily identified by PAGE analysis. However, modern methods have been used to map such interactions [6,42–44,5]. For example, Stros and Kolibalova used EDC and dimethyl-3,3′-dithiobispropionimidate to study interactions of non-histone chromosomal proteins HMG1 and HMG2 with core histones in H1, H5-depleated core particles, or nucleosomes [44]. They observed that HMG1 and HMG2 are cross-linked to core histones. The protein composition of the cross-linked conjugate was confirmed by two-dimensional SDS–PAGE analysis. In addition, no inter-nucleosomal cross-linking occurred when HMG2 was excluded from the reaction mixture. These experiments demonstrate the existence of interactions between HMG1 and HMG2 with core histones, implicating a possible role of HMG1 and HMG2 in the function and structure of chromatin.

Kumar and Davidson studied interactions between methylamine dehydrogenase and its physiological electron acceptor, amicyanin, using EDC as a cross-linker [43]. Methylamine dehydrogenase is an oligomer with an $\alpha_2\beta_2$ structure consisting of two

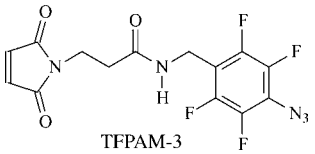


Fig. 1

large (46700 Da) and two small (15500 Da) subunits. Incubation of methylamine dehydrogenase with EDC gives only α−β cross-links [(A)–(B)]. This was consistent with crystal structures of the enzyme. Incubation of methylamine dehydrogenase with EDC in the presence of amicyanin showed formation of a conjugate that contains the large subunit of the enzyme and amicyanin, indicating that there is significant interaction in the large subunit. The dependence of the cross-linking reaction on the ionic strength of the reaction medium indicates that interactions are stabilized through hydrophobic interactions.

We have noted that von Hippel mapped protein–protein interactions in the multiprotein complex, T4 DNA polymerase [6]. He introduced cysteine at a specific site in the protein that is gene product 45 (gp45) and then attached a fluorescent label to that cysteine. In order to determine the position in the complex of gp45 relative to other gene products, he utilized the reagent *N*-(4-azido-2,3,5,6-tetrafluorobenzyl)-3-maleimidylpropionamide (TFPAM-3) (Fig. 1), which is photoactivated after addition, producing an interprotein cross-link. Benkovic extended this method significantly by utilizing a biotin ligand on the linker for identification of cross-linked units [10].

Protocol: cross-linking proteins with the photoreagent TFPAM-3 [6]

Materials

Stock solution of 4 mM TFPAM-3 (from Molecular Probes, Inc.) in DMSO
Cysteine labelling buffer: 50 mM Mops buffer (pH 7.0), 0.5 mM EDTA, and 10% glycerol
Stop solution: 1 mM cysteine
Conjugating buffer: 25 mM Hepes (pH 7.5), 60 mM potassium acetate, 6 mM magnesium acetate, and 10% glycerol
BioSpin 6 centrifuge columns from BioRad
Six-watt UV lamp (UVP, Inc., model UVL-56, Blak-Ray lamp)

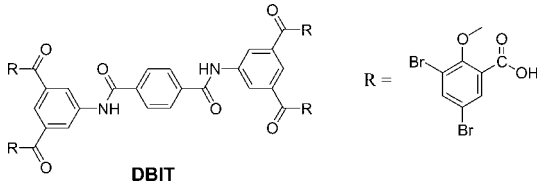
Methods

1. Pass proteins through a BioSpin 6 centrifuge column (BioRad) equilibrated with cysteine labelling buffer to remove thiols and primary amines present in storage buffer.
2. Mix 40 μM of a protein with 250 μM TFPAM-3 at room temperature for 1 h with gentle stirring.
3. Quench the reaction with 1 mM cysteine for 30 min at room temperature with gentle stirring.

4. Remove excess TFPAM-3 by passing the sample through column equilibrated with conjugating buffer. Proteins can be stored in this buffer at −20 °C.
5. Mix 1 μM of a protein labelled with TFPAM-3 with 1 μM of a protein of interest in a microcentrifuge tube (1.5 mL) in conjugating buffer.
6. Irradiate the samples for 45 min at room temperature at a distance of about 2 cm from the UV lamp. (Under these conditions label is completely photolyzed in about 35 min.)
7. Analyse products by SDS–PAGE and western blots.

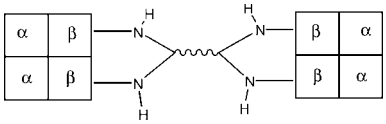
6.4. Cross-linking within and between noncomplexed proteins—synthetic complexation (A–B)–(A–B)

The use of non-specific reagents, such as glutaraldehyde, to produce cross-links utilizes a large excess of reagent. This also produces connections between proteins that are in nearby parts of the solution but are not part of a complex. Recently, we reported a specific "super-linker" (see DBIT, below) that creates cross-links within hemoglobin and also connects two hemoglobin tetramers to one another [4].



DBIT

Reaction of DBIT with deoxyhemoglobin produced a cross-linked bis-tetramer, shown schematically below. This is an (A–B)–(A–B) modification in our systematic designation scheme.



The hemoglobin is prepared for modification as it is for cross-linking and DBIT is added as a solid. The analysis is considerably more complex since the cross-linked bis-protein species is twice as large as the usual well-characterized stabilized hemoglobin. However, with size exclusion chromatography and mass spectrometry, the total mass is established. Digests permit mapping the sites of links.

Protocol: cross-linking and connecting two hemoglobins with DBIT [4]

The cross-linking reagent is not commercially available but is readily prepared.

Materials

Bis(3,5-dicarboxyphenyl)terephthalamide
tert -butyl 3,5-dibomosalicylate

Potassium *tert* -butoxide
Thionyl chloride "CAUTION" (corrosive, lachrymator, harmful liquid, combustible liquid, moisture-sensitive, refrigerate)
Hemoglobin
0.1 M sodium borate buffer, pH 9.0
0.1 M (3-[*N*-morpholino]propanesulfonic acid) (Mops) buffer, pH 7.2
TFA, trifluoro acetic acid, "CAUTION" (corrosive)
DBIT
CO, "CAUTION" (extremely flammable, very toxic)
Oxygen (compressed gas from cylinder)

Methods

Synthesis of the cross-linking reagent:

1. Bis(3,5-dicarboxyphenyl)terephthalamide (2.0 g) is stirred and heated to reflux in 10 mL of thionyl chloride for 20 h under nitrogen, and then solvent is removed in vacuo to give a white solid, which is stirred in 20 mL of anhydrous tetrahydrofuran.
2. The solution is added dropwise to a solution of *tert* -butyl 3,5-dibromosalicylate (5.8 g) and potassium *tert*-butoxide (1.9 g) in 60 mL of anhydrous tetrahydrofuran. The mixture is stirred at room temperature for 20 h under nitrogen.
3. The solid is dissolved in 50 mL of diethylether, the organic layer is washed with distilled water (3×100 mL) and dried with magnesium sulfate.
4. Remove the solvent and dissolve the remaining yellow liquid in 50 mL of TFA and stir at room temperature under nitrogen for 2 h.
5. After 2 h add ether and stir for 1 h at room temperature under nitrogen.
6. Filter the mixture and collect the product.

Cross-linking and connecting hemoglobins with DBIT

1. Pass carbonmonoxyhemoglobin (1.2 μmol) through a Sephadex G-25 column equilibrated with 50 mM sodium borate buffer, pH 9.0.
2. Bubble oxygen through the solution of hemoglobin, illuminating with visible light from a tungsten lamp, on ice for 2 h to remove the CO.
3. Deoxygenate hemoglobin by passing nitrogen through the solution at 37 °C for 2 h.
4. Add the DBIT as a solid (2.4 μmol) to the deoxyhemoglobin solution and mix gently at 37 °C for 24 h under nitrogen. (Avoid exposure of deoxyhemoglobin to air to prevent methemoglobin formation.)
5. After 24 hr cool the reaction in ice, convert to COHb by passing CO through the solution for 10 min with mixing.
6. Pass the solution of COHb cross-linked with DBIT though a Sephadex G-25 column equilibrated with 0.1 M Mops buffer, to remove unreacted DBIT.

6.5. Cross-linking between different protein complexes (AB)–(XY)

In addition to investigating protein–protein interactions within the same protein complex, chemical cross-linking has been used to investigate spatial arrangement of proteins within aggregates that contain multiple protein complexes, called super-complexes. Examples include the ATP synthase and quinol oxidase multi-complexes. ATP synthase is a multi-subunit enzyme that couples a proton gradient across cell membrane to ATP synthesis. The enzyme consists of water insoluble F_0 complex whose composition is species-dependent. For example, *Escherichia Coli* F_0 complex consists of three subunits designated *a*, *b*, and *c*, while mitochondrial ATP F_0 is more complex and may consists of up to 12 proteins. The F_1 complex consists of five different subunits $\alpha_3\beta_3\gamma\delta\varepsilon$. The F_0 and F_1 complexes are connected by a stalk region that consists of at least two proteins, oligomycin-sensitivity-conferring protein (OSCP) and coupling factor F_6. Although many of the proteins that comprise F_1–F_0 complex have been identified and cloned, their spatial arrangement, function, and stoichiometry are being studied. Joshi and Burrows analysed the spatial arrangement and subunit organization of H^+-ATPase using chemical cross-linking with the cleavable reagents dithiobis(succinimidyl propionate) (DSP) and disuccinimidyl tartrate (DST) [45]. Two-dimensional SDS–PAGE analysis and western blot analysis showed formation of several cross-linked intra- and inter-complex conjugates when H^+-ATPase was incubated with DSP. They showed that the 24 kDa protein of F_0 is cross-linked to OSCP of the stalk region. In addition, the F_6 protein of the stalk region was cross-linked to the 24 and 20 kDa proteins of F_0. The 24 and 20 kDa proteins of the F_0 complex are also cross-linked to each other. Within the F_1 complex cross-linked species consisting of γ and δ, and γ and ε subunits were characterized. This was the first observation of association between γ and δ subunits. Based on these observations a new model for subunit organization of H^+-ATPase was proposed.

Protocol: crosslinking ATP synthase complex from bovine mitochondria with DSP, dithiobis(succinimidyl propionate) [45]

Materials

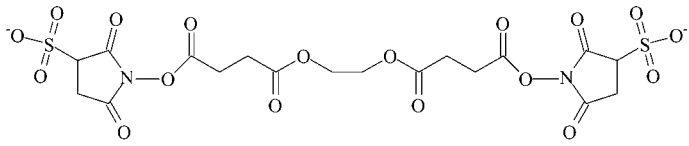
F_0, F_1-ATPase, and F_6 from bovine heart mitochondria
50 mM triethanolamin HCl, pH 8.0
Stock solution of DSP in methanol : acetone (1 : 1)
Stop solution: 125 mM Tris-HCl, pH 6.8, 4 % SDS, 4 mM EDTA, 20% glycerol

Methods

1. Prepare protein samples (1.5 mg/mL) in 50 mM triethanolamine HCl, pH 8.0, buffer containing 0.25 M sucrose.
2. Incubate a protein sample (25μg) with 1% by volume of DSP (150 μM) at 0 °C for 30 min.

3. Quench cross-linking reaction by addition of lysine to 5 mM final concentration. If preservation of enzymatic activity is not essential, the reaction can be quenched with stop buffer. (Crosslinks of proteins can be reversed by incubating the treated proteins with 80 mM 2-mercaptoethanol or 10 mM DTT.)
4. Analyse products by one- or two-dimensional SDS–PAGE or western blotting.

Quinol oxidase is another super-complex whose subunit arrangement was investigated by chemical cross-linking. Quinol oxidase from thermophilic *Bacilli* consists of two complexes: quinol-cytochrome *c* reductase and cytochrome *c* oxidase. The reductase is composed of three proteins: cytochrome b_6, cytochrome c_1, and FeS protein, while cytochrome *c* oxidase consists of four subunits (CO_1–CO_4). Tanaka et al. [46] used three cross-linking reagents DSP, DST, and sulfo-ethylene glycobis(sulfo-succinimidylsuccinate) (sulfo-EGS) to investigate the spatial relationship of subunits in the quinol oxidase super-complex.



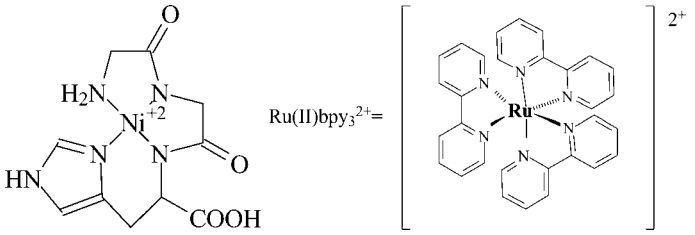
Sulfo-EGS

Two-dimensional SDS–PAGE analysis of the cross-linked species formed in the reaction of the super-complex with DST revealed a number of intra- and inter-complex cross-linked species whose components were determined. The most prominent inter-complex conjugates formed were CO_1-cytochrome b_6, CO_2–cytochrome b_6, and CO_2–cytochrome c_1.

6.6. Recent advances in chemical cross-linking for studying protein–protein interactions

Metal-catalysed oxidation is being used to cross-link proteins, a concept originally introduced by Brown et al. [47] who used a Ni(II)-peptide chelate in the presence of peracid to couple proteins. In the presence of a peroxyacid, Ni(II) is oxidized to a reactive species that can abstract electrons from adjacent aromatic residues, such as tyrosine. The resulting tyrosyl radical can be quenched by a nearby tyrosine or cysteine side chain to form a cross-link between two residues. This type of chemistry has since been used to study protein–protein interactions on a genetic level [48], as well as localized cross-linking of ribonuclease to itself [49]. More recently, Fancy and Kodadek reported cross-linking UvsY, a hexamer involved in phage T4 recombination, using a new class of photo-activable cross-linking reagent $Ru(II)bpy_3^{2+}$ [5].

The rationale is that photolysis of Ru(II)bpy_3^{2+} produces an excited state of Ru(III), which is a potent one-electron oxidant. It can oxidize residues, resulting in a cross-link similar to Brown's Ni(II)-peptide reagent. Fancy and Kodadek used Ru(II)bpy_3^{2+} to study interactions between the core domain of Gal14 (AAD), a protein that is involved in regulation of genes for metabolism of galactose, and the C-terminal region of TBP, a transcription factor that recognizes TATA element of a promoter. Photo irradiation of mixture containing Ru(II)bpy_3^{2+}, TBP, and Gal14 fusion protein generated conjugates of TBP and Gal14 fusion protein in approximately 65% yield. The extent of cross-linking can be controlled by inclusion of exogenous electron-rich compounds, such as histidine or tyrosine.



Protocol: cross-linking proteins with the photo-reagent Ru(II)bpy_3 Cl_2 [5]

Materials

Ru(II)bpy_3Cl_2 stock solution
Conjugating buffer: 15 mM sodium phosphate (pH 7.5), 150 mM NaCl.
Ammonium persulfate
Stop solution: 0.2 M Tris, 8% SDS, 2.88 M β-mercaptoethanol, 40% glycerol, 0.4% xylene cyanol, and 0.4% bromophenol blue buffer
Xenon arc lamp (150 W) (Oriel, Stamford, CT) and 380–2 500 nm cut-on filter Oriel 49470
Optional: single-lens reflex camera with the lens and back cover removed

Methods

1. Mix proteins (0.01–20 μM) with the reaction buffer consisting of 15 mM sodium phosphate (pH 7.5), 150 mM NaCl, and 0.125 mM Ru(II)bpy_3Cl_2. DTT and β-mercaptoethanol should be excluded from the reaction buffer as they can be oxidized in the reaction.
2. Place the solution in a 1.7 mL Eppendorf tube parallel to the beam of light at a distance of 50 cm from a 150 W xenon arc lamp (Oriel, Stamford, CT), or at a distance of 5 cm for the high-intensity standard flashlight. (For xenon lamp, the light should be filtered first through 10 cm of distilled water, and then through a 380-2 500 nm cut-on filter Oriel 49470.)
3. Add ammonium persulfate to 2.5 mM concentration just before irradiation.

4. Irradiate sample for 0.5 s for xenon lamp, and for 5–30 s for flashlight. (For xenon lamp, exposure time can be controlled by shining light through the shutters of a single lens reflex camera with the lens and back cover removed.)
5. Quench the reaction with approximately 10 μL of 0.2 M Tris, 8% SDS, 2.88 M β-mercaptoethanol, 40% glycerol, 0.4% xylene cyanol, and 0.4% bromophenol blue buffer.
6. Analyse products by SDS–PAGE and visualize by staining with Coomassie brilliant blue or by Western blot analysis.

6.7. Future directions

The use of cross-linking to learn about complex structures is a rapidly developing area of interest and results come from many directions. New classes of reagents that can connect efficiently within and between entities make these methods a significant approach for learning about structure and function. The ability to produce controlled arrays can lead to information about effects in transient as well as stable interactions. The increasing sensitivity, wider range, and database analysis of mass spectrometry will enable more structures to be solved rapidly. The increasing use of photo-activated reagents and affinity recognition with biotin–streptavidin provide a means of creating and analysing links that trap interactions without needing to know any structural details. We anticipate that structural definition in the cross-link and selectivity in the reagents will enable more materials to be prepared. In addition, methods of introducing conjugates will permit signals to be permanently connected. The chemical synthesis involved in producing new directions in this area remains surprisingly simple to accomplish but the convenience and reliability of commercial sources has led to the suppliers having a large say in the direction of the field. The interdisciplinary nature of the methods necessitates fruitful collaborations, a very positive by-product of modern science. Protein–protein interactions are ubiquitous but difficult to detect. Mapping their coordinates will provide us with an important guide to dynamic structure and function.

Acknowledgment

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References

[1] J. Deisenhofer, O. Epp, K. Miki, R. Huber, H. Michel, J. Mol. Biol. 180 (1984) 385–398.
[2] J. Deisenhofer, H. Michel, EMBO J. 8 (1989) 2149–2170.

[3] R. Uy, F. Wold, Protein Crosslinking, Plenum, New York, 1977.
[4] R. Kluger, J.L. O'Brien, A. Teytelboym, J. Am. Chem. Soc. 121 (1999) 6785–7680.
[5] D.A. Fancy, T. Kodadek, Proc. Natl. Acad. Sci. USA 96 (1999) 6020–6024.
[6] P. Pietroni, M.C. Young, G.J. Latham, P.H. von Hippel, J. Biol. Chem. 272 (1997) 31666–31676.
[7] R.P. Anthony, D.T. Brown, J. Virol. 65 (1991) 1187–1194.
[8] X. Chen, D. Eswaran, M.A. Smith, G. Perry, V.E. Anderson, Bioconjug. Chem. 10 (1999) 112–118.
[9] X. Chen, Y.H. Chen, V.E. Anderson, Anal. Biochem. 273 (1999) 192–203.
[10] C.S. Alley, F.T. Ishmael, A.D. Jones, S.J. Benkovic, J. Am. Chem. Soc. 122 (2000) 6126–6127.
[11] R.A. Smith, J.R. Knowles, Biochem. J. 141 (1974) 51–55.
[12] D.N. Standring, J.R. Knowles, Biochemistry 19 (1980) 2811–2816.
[13] J.V. Staros, J.R. Knowles, Biochemistry 17 (1978) 3321–3325.
[14] H. Bayley, J.R. Knowles, Methods Enzymol. 46 (1977) 69–114.
[15] V. Chowdhry, F.H. Westheimer, Annu. Rev. Biochem. 48 (1979) 293–325.
[16] V. Chowdhry, R. Vaughan, F.H. Westheimer, Proc. Natl. Acad. Sci. USA 73 (1976) 1406–1408.
[17] J.P. Weber, S.A. Bernhard, Biochemistry 21 (1982) 4189–4194.
[18] D.K. Srivastava, S.A. Bernhard, Biochemistry 23 (1984) 4538–4545.
[19] D.K. Srivastava, S.A. Bernhard, Curr. Top. Cell. Regul. 28 (1986) 1–68.
[20] J. Rudolph, J. Stubbe, Biochemistry 34 (1995) 2241–2250.
[21] T.H. Ji, Methods Enzymol. 91 (1983) 580–606.
[22] K. Martinek, V.P. Torchilin, Methods Enzymol. 137 (1988) 615–626.
[23] Pierce Chemical Company, Catalog and Handbook, Rockford, IL, 2004.
[24] G.T. Hermanson, Bioconjugate Techniques, Academic Press, San Diego, CA, 1996.
[25] R. Uy, F. Wold, Adv. Exp. Med. Biol. A 86 (1977) 169–186.
[26] R. Kluger, in: T.E. Creighton (Ed.), Protein Function, A Practical Approach, URL Press, Oxford, 1997, pp. 185–214.
[27] R. Kluger, L. Shen, H. Xiao, R.T. Jones, J. Am. Chem. Soc. 118 (1996) 8782–8786.
[28] M.A. Schumacher, M.M. Dixon, R. Kluger, R.T. Jones, R.G. Brennan, Nature 375 (1995) 84–87.
[29] G.E. Means, R.E. Feeney, Chemical Modification of Proteins, Holden-Day, San Francisco, LA, 1971.
[30] R. Kluger, Synlett (2000) 1708–1720.
[31] R. Kluger, V.D. Stefano, J. Org. Chem. 65 (2000) 214–219.
[32] R. Kluger, Y. Song, J. Wodzinska, C. Head, T.S. Fujita, R.T. Jones, J. Am. Chem. Soc. 114 (1992) 9275–9279.
[33] R. Kluger, J. Wodzinska, R.T. Jones, C. Head, T.S. Fujita, D.T. Shih, Biochemistry 31 (1992) 7551–7559.
[34] Pierce Chemical Company, Catalog and Handbook, Rockford, IL, 2000.
[35] R. Kluger, S.L. Bearne, Bioorg. Med. Chem. 2 (1994) 379–385.
[36] R. Kluger, Y. Song, J. Org. Chem. Soc. 59 (1994) 733–736.
[37] R.T. Jones, Methods Enzymol. 231 (1994) 322–343.
[38] M. Burke, E. Reisler, Biochemistry 16 (1977) 5559–5563.
[39] L.K. Nitao, E. Reisler, Biochemistry 37 (1998) 16704–16710.
[40] L.K. Nitao, E. Reisler, Biophys. J. 78 (2000) 3072–3080.
[41] S. van Dijk, D.N. Ward, Endocrinology 132 (1993) 534–538.
[42] T.C. Squier, S.E. Hughes, D.D. Thomas, J. Biol. Chem. 263 (1988) 9162–9170.
[43] M.A. Kumar, V.L. Davidson, Biochemistry 29 (1990) 5299–5304.
[44] M. Stros, A. Kolibalova, Eur. J. Biochem. 162 (1987) 111–118.
[45] S. Joshi, R. Burrows, J. Biol. Chem. 265 (1990) 14518–14525.
[46] T. Tanaka, M. Inoue, J. Sakamoto, N. Sone, J. Biochem. (Tokyo) 119 (1996) 482–486.
[47] K.C. Brown, S.H. Yang, T. Kodadek, Biochemistry 34 (1995) 4733–4739.
[48] K.C. Brown, Z. Yu, A.L. Burlingame, C.S. Craik, Biochemistry 37 (1998) 4397–4406.
[49] G. Gill, A.A. Richter-Rusli, M. Ghosh, C.J. Burrows, S.E. Rokita, Chem. Res. Toxicol. 10 (1997) 302–309.