TEACHING EDITORIAL

A Brief Survey of Methods for Preparing Protein Conjugates with Dyes, Haptens, and Cross-Linking Reagents

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I. INTRODUCTION

Modification of proteins, DNA, and other biopolymers by labeling them with reporter molecules has become a very powerful research tool in immunology, histochemistry, and cell biology. A number of excellent reviews of this subject have been published (*1–6*). In addition, there are a growing number of commercial applications of these modified biomolecules, including clinical immunoassays, DNA hybridization tests, and gene fusion detection systems. In these techniques, a small molecule with special properties, such as fluorescence or binding specificity, is covalently bound to a protein, a DNA strand, or other biomolecule. Specific examples include fluorescent-labeled antibodies for detection and localization of cell-surface antigens, biotin-labeled single-stranded DNA probes for detection of DNA hybridization, and hapten-labeled proteins that, when introduced into a suitable host animal, generate hapten-specific antibodies.

This review will focus on the experimental design and procedures for preparing protein conjugates with dyes, biotin, and haptens such as drugs and hormones. Methods for covalently linking two unlike biopolymers through the judicious choice of cross-linking reagents will also be discussed. The following specific topics will be addressed: (a) reactive groups of proteins that are available for modification, including their naturally occurring amino acids, and reactive groups introduced by chemical modification, (b) reagents that can be used to couple molecules to these reactive sites, (c) experimental procedures for preparing conjugates, (d) purification and isolation of conjugates, and (e) techniques for determining the degree of labeling.

II. GENERAL DISCUSSION OF METHODS

A. Reactive Groups of Proteins. Proteins and peptides are amino acid polymers containing a number of reactive side chains. In addition to, or as an alternative to, these intrinsic reactive groups, specific reactive moieties can be introduced into the polymer chain by chemical modification. These groups, whether or not they are naturally a part of the protein or are artificially introduced, serve as "handles" for attaching a wide variety of molecules, including other proteins. The intrinsic reactive groups of proteins are described in the following section.

(*1*) *Amines* (*Lysines*, *α-Amino Groups*). One of the most common reactive groups of proteins is the aliphatic ϵ-amine of the amino acid lysine. Lysines are usually present to some extent and are often quite abundant. For example, the protein bovine insulin contains only a single lysine amine, while avidin, a protein found in egg whites, contains 36 lysines (*7*). Lysine amines are reasonably good nucleophiles above pH 8.0 (pK_a = 9.18) (*8*) and therefore stable bonds (eq 1). Other reactive amines that are found

$$\text{Protein-NH}_2 + \text{RX} \longrightarrow \text{Protein-NHR} + \text{XH} \quad (1)$$

in proteins are the α-amino groups of the N-terminal amino acids. The α-amino groups are less basic than lysines and are reactive at around pH 7.0. Sometimes they can be selectively modified in the presence of lysines. There is usually at least one α-amino acid in a protein, and in the case of proteins that have multiple peptide chains or several subunits, there can be more (one for each peptide chain or subunit). Bovine insulin has one N-terminal glycine residue and one N-terminal phenylalanine (*9*). There are proteins that do not possess free α-amino groups, such as cytochrome C and ovalbumin. In these molecules, the N-terminal amino group is N-acylated, and therefore not reactive toward the usual modification reagents. Since either N-terminal amines or lysines are almost always present in any given protein or peptide, and since they are easily reacted, the most commonly used method of protein modification is through these aliphatic amine groups.

(*2*) *Thiols* (*Cystine*, *Cysteine*, *Methionine*). Another common reactive group in proteins is the thiol residue from the sulfur-containing amino acid cystine and its reduction product cysteine (or half-cystine), which are counted together as one of the 20 amino acids. Cysteine contains a free thiol group, which is more nucleophilic than amines and is generally the most reactive functional group in a protein. It reacts with some of the same modification reagents as do the amines discussed in the previous section and in addition can react with reagents that are not very reactive toward amines. Thiols, unlike most amines, are reactive at neutral pH, and therefore they can be coupled to other molecules selectively in the presence of amines (eq 2). This selectivity makes the thiol

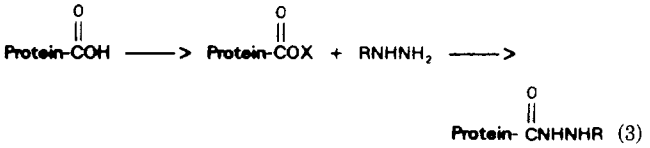
$$\text{NH}_2\text{-Protein-SH} + \text{RX} \longrightarrow \text{NH}_2\text{-Protein-SR} + \text{XH} \quad (2)$$

group the linker of choice for coupling two proteins together, since methods which only couple amines (e.g., glutaraldehyde, dimethyl adipimidate coupling) can result in formation of homodimers, oligomers, and other unwanted products (*10*). Since free sulfhydryl groups are relatively reactive, proteins with these groups often exist in their oxidized form as disulfide-linked oligomers or have internally bridged disulfide groups. Immunoglobulin M is an example of a disulfide-linked pentamer, while immunoglobulin G is an example of a protein with internal disulfide bridges bonding the subunits together. In proteins such as this, reduction of the disulfide bonds with a reagent such as dithiothreitol (DTT) is required to generate the reactive free thiol (*11*). In addition to cystine and cysteine, some proteins also have the amino acid methionine, which contains sulfur in a thioether linkage.

with thiol-reactive reagents such as iodoacetamides (*12*). However, selective modification of methionine is difficult to achieve and therefore is seldom used as a method of attaching small molecules to proteins.

(*3*) *Phenols* (*Tyrosine*). The phenolic substituent of the amino acid tyrosine can react in two ways. The phenolic hydroxyl group can form esters and ether bonds, and the aromatic ring can undergo nitration or coupling reactions with reagents such as diazonium salts at the position adjacent to the hydroxyl group. There is considerable literature describing the reaction of tyrosyl residues with diazonium compounds (*13*). For example, a *p*-aminobenzoyl biocytin derivative has been diazotized and reacted with protein tyrosine groups (*14*). Modification of tyrosines has primarily been used in structural studies, rather than as a means for attaching specific labels, since acetylation and nitration can give useful information concerning the participation of tyrosine in the binding properties of proteins. Often, the reactivity of tyrosines with amine-selective modification reagents to form unstable carboxylic acid esters or sulfate esters is an unwanted side reaction resulting in conjugates that slowly hydrolyze during storage. Methods for preventing this problem are discussed in a later part of this teaching editorial (section V.B.1).

(*4*) *Carboxylic Acids* (*Aspartic Acid*, *Glutamic Acid*). Proteins contain carboxylic acid groups at the carboxy-terminal position and within the side chains of the dicarboxylic amino acids aspartic acid and glutamic acid. The low reactivity of carboxylic acids in water usually makes it difficult to use these groups to selectively modify proteins and other biopolymers. In the cases where this is done, the carboxylic acid group is usually converted to a reactive ester by use of a water-soluble carbodiimide



(3)

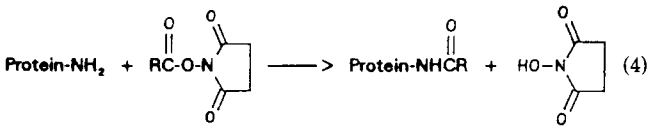
and then reacted with a nucleophilic reagent such as an amine or a hydrazide (*15*, *16*). The amine reagent should be weakly basic in order to react specifically with the activated carboxylic acid in the presence of the other amines on the protein. This is because protein cross-linking can occur when the pH is raised to above 8.0, the range where the protein amines are partially unprotonated and reactive. For this reason, hydrazides, which are weakly basic, are useful in coupling reactions with a carboxylic acid (*17*). This reaction can also be used effectively to modify the carboxy terminal group of small peptides.

(*5*) *Other Amino Acid Side Chains* (*Arginine*, *Histidine*, *Tryptophan*). Chemical modification of other amino acid side chains in proteins has not been extensive, compared to the groups discussed above. The high pK_a of the guanidine functional group of arginine (pK_a = 12–13) necessitates more drastic reaction conditions than most proteins can survive. Arginine modification has been accomplished primarily with glyoxals and α-diketone reagents (*18*). Tryptophan modification requires harsh conditions and is seldom carried out except as a method of analysis in structural or activity studies. Histidines have been subjected to photooxidation (*19*) and reaction with iodoacetates (*20*).

B. Protein Modification Reagents. This section will for the purpose of protein modification. The fundamental principles for understanding how to use these reagents are (1) recognition of the reactive group(s) on the protein or peptide that can be modified and (2) knowledge of the type of chemical reactions these reactive groups will participate in and the nature of the chemical bonds that will result from these reactions.

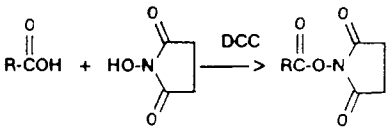
(*1*) *Amine-Reactive Reagents*. These reagents are those which will react primarily with lysines and the α-amino groups of proteins and peptides under both aqueous and nonaqueous conditions. Some amine-reactive reagents are more reactive, and therefore less selective, than others, and it will be necessary to understand this property in order to choose the best reagent for modification of a specific protein. The following amine-reactive reagents are available.

(*a*) *Reactive Esters* (*Formation of an Amide Bond*). Reactive esters, especially *N*-hydroxysuccinimide (NHS) esters, are among the most commonly used reagents for modification of amine groups (*21*). These reagents have intermediate reactivity toward amines, with high selectivity toward aliphatic amines. Their reaction rate with aromatic amines, alcohols, phenols (tyrosine), and histidine is relatively low. Reaction of NHS esters with amines under nonaqueous conditions is facile, so they are useful for derivatization of small peptides and other low molecular weight biomolecules. The optimum pH for reaction in aqueous systems is 8.0–9.0. The aliphatic amide products which are formed are very stable (eq 4). The



(4)

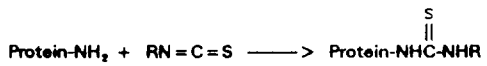
NHS esters are slowly hydrolyzed by water (*22*), but are stable to storage if kept well desiccated. Virtually any molecule that contains a carboxylic acid or that can be chemically modified to contain a carboxylic acid can be converted into its NHS ester (eq 5), making these reagents



(5)

among the most powerful protein-modification reagents available. Newly developed NHS esters are available with sulfonate groups that have improved water solubility (*23*). A short list of reactive NHS ester derivatives of fluorescent probes, biotin, and other molecules is given in Table I.

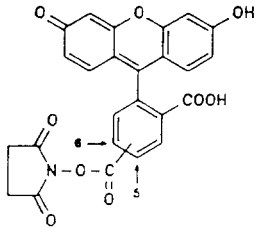
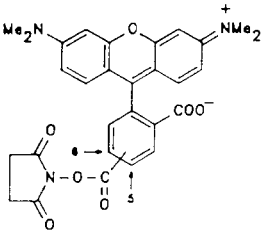
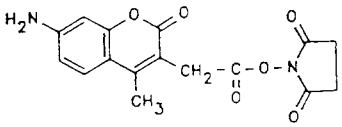
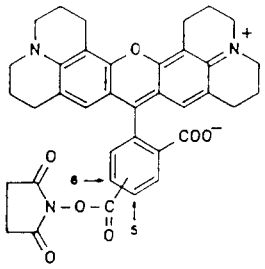
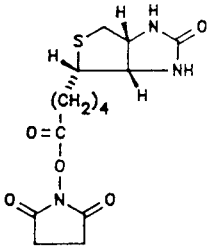
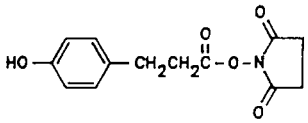
(*b*) *Isothiocyanates* (*Formation of a Thiourea Bond*). Isothiocyanates, like NHS esters, are amine-modification reagents of intermediate reactivity and form thiourea bonds with proteins and peptides (eq 6). They are



(6)

somewhat more stable in water than the NHS esters and react with protein amines in aqueous solution optimally at pH 9.0–9.5. Since this is a higher pH than the optimal pH for NHS esters (which undergo competing hydrolysis at pH 9.0–9.5), isothiocyanates may not be as suitable as NHS esters when modifying proteins that are sensitive to alkaline pH conditions. One of the most commonly used fluorescent derivatization reagents for proteins is fluo-

Table I. Succinimidyl Ester Probes

probes	structure	function	ref
succinimidyl fluorescein-5-(and -6-)carboxylate		fluorescent label	75, 76
succinimidyl *N,N,N′,N′*-tetramethylrhodamine-5-(and -6-)carboxylate		fluorescent label	76
succinimidyl 7-amino-4-methylcoumarin-3-acetate		fluorescent label	77
succinimidyl X-rhodamine-5-(and -6-)carboxylate		fluorescent label	75, 78
succinimidyl D-biotin		ligand, affinity label	79
succinimidyl 3-(4-hydroxyphenyl)propionate		radioiodination label	80

rescent dyes (coumarins and rhodamines) have been coupled to proteins via their reactive isothiocyanates (*24*).

(*c*) *Aldehydes* (*Formation of Imine, Reduction to Alkylamine Bond*). Aldehyde groups react under mild aqueous conditions with aliphatic and aromatic amines to form an intermediate known as a Schiff base (an imine), which can be selectively reduced by the mild reducing agent sodium cyanoborohydride to give a stable alkylamine bond (eq 7) (*44*, *53*). This method of amine modification is not used

$$\text{Protein-NH}_2 + \text{RCH=O} \longrightarrow \text{Protein-N=CHR} \xrightarrow{\text{NaBH}_3\text{CN}} \text{Protein-NHCH}_2\text{R} \quad (7)$$

in protein conjugations as frequently as the activated ester method, but when the molecule to be attached has an

hyde, the method is mild, simple, and very effective. Aldehydes (glyoxals) can also react with protein arginine groups (*25*, *26*) and the nucleic acid base guanosine, making them of some use in nucleic acid modification (*27*).

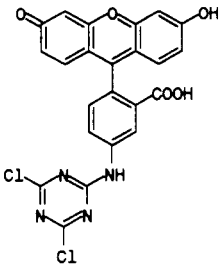
(*d*) *Sulfonyl Halides* (*Formation of a Sulfonamide Bond*). Sulfonyl halides are highly reactive amine-modifying reagents. They are unstable in water, especially at the pH required for reaction with aliphatic amines, but they form extremely stable sulfonamide bonds which can survive even amino acid hydrolysis (eq 8). It is for this

$$\text{Protein-NH}_2 + \text{R-S(=O)}_2\text{-Cl} \longrightarrow \text{Protein-NH-S(=O)}_2\text{-R} + \text{HCl} \quad (8)$$

reason that sulfonamide conjugates are useful for

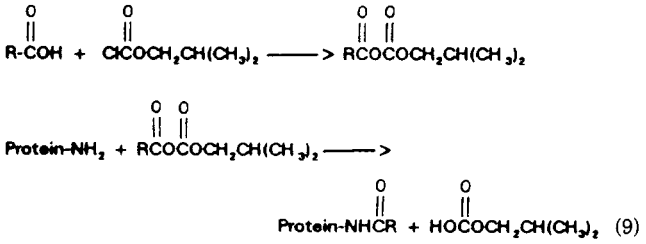
as tracers (*28*). In addition to amines, sulfonyl halides also react with phenols (tyrosine), thiols (cysteine), and imidazoles (histidine) on proteins (*29*); therefore, they are less selective than either NHS esters or isothiocyanates. The conjugates formed with thiols, imidazoles, and phenols are all unstable and, if not removed during purification, can lead to loss of the label from the protein during long-term storage (see section V.B.1). One of the most widely used long-wavelength fluorescent probes, Texas Red, is a sulfonyl chloride. It has the longest wavelength spectral properties of any of the common amine-reactive fluorescent labeling reagents (*30*).

(*e*) *Miscellaneous Amine Reactive Reagents* (*Dichlorotriazines, Alkyl Halides, Anhydrides*). The dichlorotriazine derivative of fluorescein, known as DTAF (I), has



I

high reactivity with protein amines and has been used to prepare fluorescein tubulin with minimal loss of activity (*31*). In addition to amines, dichlorotriazines will react with alcohols at elevated temperatures (60–90 °C) and are used to prepare polysaccharide conjugates (*32*). Some alkyl halides, including iodoacetamides commonly used to modify thiols, will react with amines of proteins if the pH is in the range 9.0–9.5 (*33*). Other reagents that have been used to modify amines of proteins are acid anhydrides. Succinic anhydride is commonly used to succinylate amine groups of basic proteins for the purpose of changing their isoelectric point and other charge-related properties (*34*). Mixed anhydrides derived from reaction of a carboxylic acid with carbitol or 2-methylpropanol chloroformates (eq 9) are excellent reagents for modification of amines under

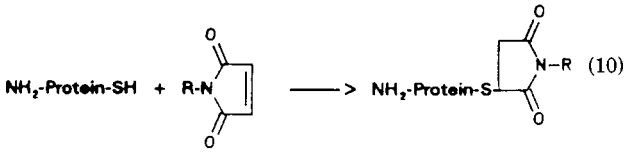


mild conditions (*35*). Of these, the carbitol mixed anhydride is relatively water soluble and is the preferred reagent for modification of amines in aqueous solution.

(*2*) *Thiol-Reactive Reagents*. Thiol-reactive reagents are those that will couple to thiol groups on proteins to give thioether-coupled products. These reagents react rapidly at neutral (physiological) pH and therefore can be reacted with thiols selectively in the presence of amine groups.

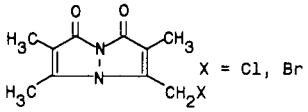
(*a*) *Haloacetyl Derivatives* (*Formation of a Thioether Bond*). These reagents (usually iodoacetamides) are among the most frequently used reagents for thiol modification. In most proteins, the site of reaction is at cysteine residues that result from reduction of cystines. The reaction of iodoacetate with cysteine is approximately twice as fast as that with bromoacetate and 20–100 times as rapid as that with chloroacetate (*36*). As mentioned previously, in the absence of cysteines, methionines can sometimes react with haloacetamides (*12*). Reaction of haloacetamides with thiols occurs rapidly at neutral pH at room temperature or below, and under these conditions, most aliphatic amines are unreactive. In addition to proteins, haloacetamides have been reacted with thiolated peptides and thiolated primers for DNA sequencing (*37*), and also with RNA (on thiouridine) (*38*). The thioether linkages formed from reaction of haloacetamides are very stable. A potential problem in using iodoacetamides as modification reagents is their instability to light, especially in solution; therefore, they must be protected from light in storage and during reaction. The fluorescein and rhodamine iodoacetamides are among the most intensely fluorescent sulfhydryl reagents available for protein and peptide modification.

(*b*) *Maleimides* (*Formation of a Thioether Bond*). Maleimides (eq 10) are similar to iodoacetamides in their



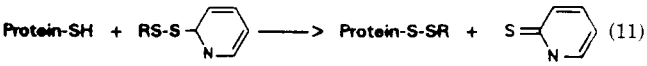
application as reagents for thiol modification; however, they are more selective than iodoacetamides, since they do not react with histidine, methionine, or thionucleotides (*39, 40*). The optimum pH for the reaction of maleimides is near 7.0. Above pH 8.0, hydrolysis of maleimides to nonreactive maleamic acids can occur (*41*).

(*c*) *Miscellaneous Thiol-Reactive Reagents*. These reagents include bromomethyl derivatives and pyridyl disulfides. The bromomethyl derivatives are similar in reactivity to iodoacetamides. The haloalkyl derivatives monobromobimane and monochlorobimane (II) react with

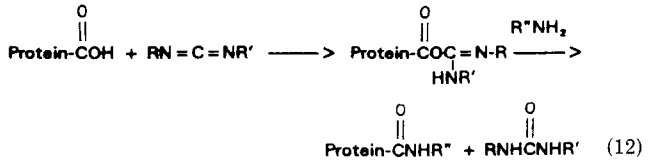


II

glutathione and other thiols in cells to give fluorescent adducts, thus providing a method of quantitation of thiols (*42*). Pyridyl disulfides react in an exchange reaction with protein thiols to give mixed disulfides (eq 11) (*43*).

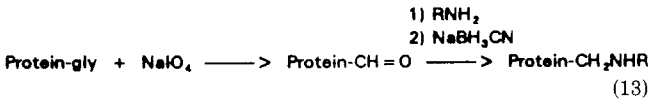


(*3*) *Carboxylic Acid- and Aldehyde-Reactive Reagents*. (*a*) *Amines and Hydrazides* (*Formation of Amide or Alkylamine Bonds*). Amines and hydrazides can be coupled to carboxylic acids of proteins via activation of the carboxyl group by a water-soluble carbodiimide followed by reaction with the amine or hydrazide. As mentioned previously (section II.A.4), the amine or hydrazide reagent must be weakly basic so that it will react selectively with the carbodiimide-activated protein in the presence of the more highly basic protein ϵ-amines (lysines). The reaction of these probes with carbodiimide-activated carboxyl groups leads to the formation of stable amide bonds (eq



(12)

Amines and hydrazides are also able to react with aldehyde groups, which can be generated on proteins by periodate oxidation of carbohydrate residues on the protein. In this case, a Schiff base intermediate is formed (eq 13), which can be reduced to an alkylamine with sodium

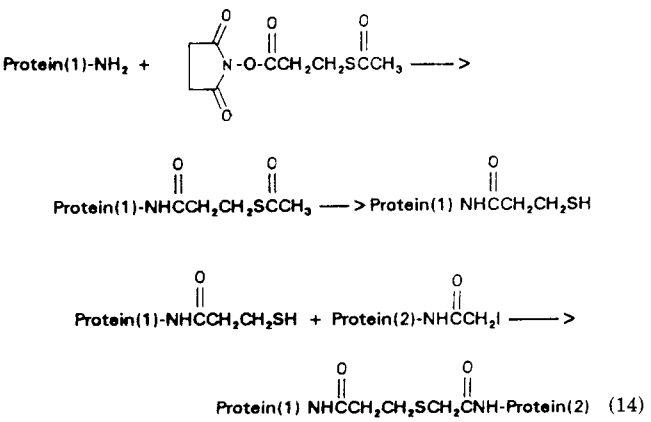


(13)

cyanoborohydride, a mild and selective water-soluble reducing agent (*44*) (see also section II.B.1.c). Since the Schiff base formation is reversible, it is possible to minimize formation of protein–protein products by adding a large excess of amine or hydrazide reagent.

(*4*) *Bifunctional Reagents.* Bifunctional, or cross-linking, reagents are specialized reagents having reactive groups that will form a bond between two different groups, either on the same molecule or two different molecules. Bifunctional reagents can be divided into two types: those with the same reactive group at each end of the molecule (homobifunctional) and those with different reactive groups at each end of the molecule (heterobifunctional). Recent trends are heavily in favor of the use of heterobifunctional cross-linkers where the bifunctional reagent has two reactive sites, each with selectivity toward different functional groups (amine reactive and thiol reactive, for example). These reagents, some of which are available in a range of chain lengths, are well-suited to the task of controlled coupling of unlike biomolecules, such as two different proteins. Table II lists some frequently used heterobifunctional cross-linkers along with their reactivities and references describing their use.

(*a*) *Amine Reactive—Thiol or Protected Thiol.* Because thiols will react selectively in the presence of amines with a variety of reagents, these functional groups are very useful for attaching two different proteins together. Thiol-coupling methods are frequently employed to prepare protein–enzyme conjugates. If the proteins to be coupled do not contain intrinsic thiols, the procedure is typically carried out by introducing a single thiol group to an amine of one of the proteins by means of a heterobifunctional reagent (eq 14). Traut's reagent (iminothiolane) has been

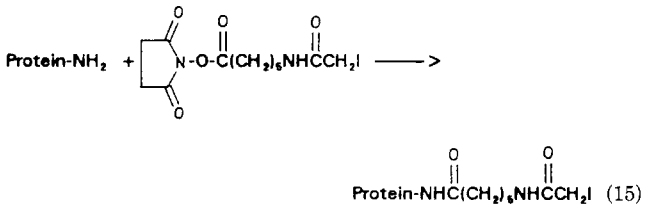


(14)

extensively used for the purpose of introducing thiol groups

reagents contain both an amine-reactive and a protected thiol group, such as succinimidyl (acetylthio)acetate (SATA) (*47*, *48*) or succinimidyl 3-(2-pyridyldithio)propionate (SPDP) (*43*, *49*). After deprotection, the thiol-containing protein is then reacted with a thiol-reactive group on the other protein, which has been introduced by a similar technique. Alternatively, proteins with synthetic thiol groups that have been introduced by modification can be used to couple to a number of thiol-reactive derivatives of dyes, biotin, haptens, or other molecules.

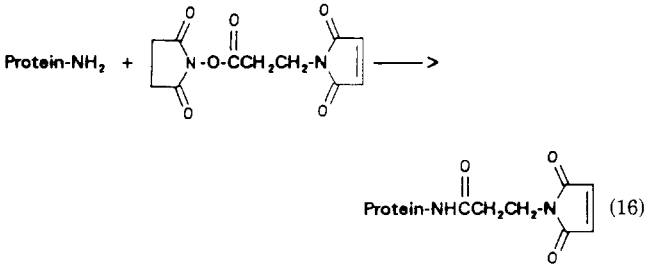
(*b*) *Amine Reactive—Iodoacetamide.* Iodoacetamides are primarily thiol-reactive groups with the reaction occurring rapidly at physiological pH, but they can react with amines under more alkaline conditions (greater than pH 9.0) and long reaction times (section II.B.2.a). Iodoacetamides can be introduced into a protein or peptide that does not have intrinsic thiols via amine-reactive derivatives (eq 15) (*50*). The resulting modified protein



(15)

can then be coupled to any thiol-containing molecule. The second molecule is usually a thiol-containing protein.

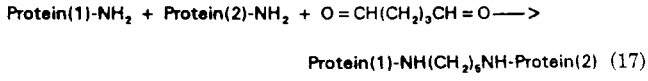
(*c*) *Amine Reactive—Maleimide.* The introduction of maleimides into a protein or peptide can be carried out with heterobifunctional reagents that have an amine-reactive group at one end and the thiol-specific maleimide at the other end (eq 16). The applications are very



(16)

similar to those for the iodoacetamides discussed in the preceding section. Specific applications include coupling of ricin to monoclonal antibodies (*51*) and linking of oligonucleotides to enzymes (*52*).

(*d*) *Amine Reactive—Aldehyde.* Aldehydes do not occur naturally in proteins, but can be introduced in two ways. In the first method, carbohydrate groups on proteins are treated with an oxidizing reagent, such as sodium periodate, or are converted via a galactose oxidase/catalase enzyme method, both of which split the sugar to form aldehyde groups (*53*). Not all proteins contain carbohydrate groups, and therefore a second method of introducing aldehydes via the reagent glutaraldehyde has been employed (*10*). Glutaraldehyde has been used extensively to couple two proteins together via their amine groups (eq 17); however, like other homobifunctional reagents, glu-



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taraldehyde is being replaced with more selective heter-

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