

Chapter 8

Identifying Residues in Antigenic Determinants by Chemical Modification

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Summary

Chemical modification of the side chains of amino acid residues was one of the first methods developed to investigate epitopes in protein antigens. The principle of the method is that alteration of the structure of a key residue of an epitope by a chemical modification will alter reactivity with antibody by affecting either specificity or avidity or both. Chemical modification has the advantage that it can be applied to discontinuous as well as continuous epitopes and may be of value in identifying cryptic epitopes. We consider here the several recent studies that have applied site-specific chemical modification to the identification of epitopes on antigens, including the use of formaldehyde, glutaraldehyde, and acid anhydrides, to produce allergoids where determinants important to reaction with IgE are modified but the ability to elicit an IgG response is retained. It is noteworthy that modification of amino groups with charge reversal appears to be the most useful approach. The approach to the use of site-specific chemical modification as a tool for the study of protein function is discussed, and emphasis is placed on the necessity to (1) validate the specificity of modification and (2) assess potential conformational change that may occur secondary to modification. Finally, a list of chemical reagents used for protein modification is presented, together with properties and references to use.

Key words: Chemical modification, Side chain modification, Antibody, Epitope mapping, Conformational epitope, Discontinuous epitope, Linear epitope.

1. Introduction

An antigenic determinant is that region of an antigen molecule, termed an epitope, that interacts with the specific antigen-binding site in the variable region of an antibody molecule which is known as a paratope (1, 2). There are a number of methods for identifying epitopes described throughout this volume, including

X-ray crystallography (3–5) (*see* Chapter “Structural Basis of Antibody–Antigen Interactions”) and phage display (6–8) (*see* Chapters “Epitope Mapping Using Phage Display Peptide Libraries,” “Humoral Response to Immunoselected Phage-Displayed Peptides by Microarray-Based Method,” and “Epitope Mapping Using Phage-Display Random Fragment Libraries”), within the developing field of structural immunology (9, 10).

Site-specific chemical modification has been useful in defining structure–function relationships in proteins (11, 12). Chemical modification of the side chains of residues in protein antigens was one of the first methods developed to investigate epitopes. Together with proteolytic fragmentation (*see* also Chapters “Proteolytic Fragmentation for Epitope Mapping” and “Epitope Mapping by Proteolysis of Antigen–Antibody Complexes”), it played a major role in the pioneering efforts of Atassi and others to assign antigenic determinants on the surfaces of lysozyme and myoglobin (13, 14). The principle of the method is that alteration of the structure of a key residue of an epitope by a chemical modification reagent will greatly change its reactivity with an antibody to that epitope. Chemical modification has the advantage that it can be applied to discontinuous as well as continuous epitopes (15, 16). Chemical modification can also be of value in identifying cryptic epitopes (17). Moreover, continuous epitopes can be conformationally constrained in the context of the folded protein and may not always adopt a recognizable conformation when removed from that context. It is usually necessary to combine chemical modification with other technologies to identify a particular residue antigen, rather than a residue type, as being a key epitope constituent. Hence, it is necessarily an adjunct to other tools that can locate the individual residue of the type being modified. The exception is when a particular type of residue occurs infrequently, *i.e.*, only once, perhaps twice, in the protein. This is more common in smaller proteins and peptides or with residues that are infrequently expressed in proteins. Examples include an analysis of the roles of histidines in the expression of Gm allotypes on IgG (18) and investigation of a cysteine residue in HLA-B27 (19). Generally, identification of the exact residue will not be achieved. Nevertheless, the range of chemical reactivities of residues has made it possible to prepare partially or even singly modified derivatives of some antigens (20, 21), which thus permits direct identification of key residues.

Neurath and others (22) used a variety of chemical reagents, including 1,2-cyclohexanedione (arginine), citraconic anhydride (amino groups including lysine), tetranitromethane (tyrosine), and carbodiimide (carboxyl groups), to identify a hepatitis B surface antigenic determinant. These investigators also used cyanogen bromide cleavage at methionine residues and 2-nitro-5-thiocyanobenzoic acid cleavage at cysteine residues. Subsequent

studies by Peterson and colleagues demonstrated that the modification of lysine residues with reductive methylation did not affect monoclonal antibody binding by the surface antigen (23). Studies by Neurath and others (24) used a different set of monoclonal antibodies to study the effect of reductive methylation and modification by citraconylation on the hepatitis B surface antigen. This study showed that reductive methylation had an effect on the surface antigenic determinant with this set of antibodies but the effect was less than that observed with citraconylation. It is important to note that modification with citraconic anhydride results in charge reversal (positive to negative) while the positive charge is maintained on reductive methylation. Neurath and co-workers have also used this technology to study the binding of a porphyrin, *meso*-teta(4-carboxyphenylporphine), to HIV-1 glycoprotein, gp120 (25, 26).

Although not directed specifically at the issue of epitope identification by chemical modification, we would be remiss in not mentioning the use of chemical modification in the production of allergoids. Allergoids are modified allergens that have decreased allergenicity but unchanged immunogenicity. Thus, the administration of allergoids would result in the production of IgG “blocking” antibodies preventing the type I hypersensitive (allergic/anaphylactoid) response (27), characterized by a humoral IgE response (28). Marsh and co-workers introduced the concept of allergoids in 1970 (29), when they demonstrated that the modification of allergens with formaldehyde resulted in the formation of allergoids. Subsequent studies have used carbamylation and modification with organic acid anhydrides (30). While recombinant allergens have been developed for this use, there is still interest in the classical chemical approach for the synthesis of allergoids (31, 32).

Site-specific chemical modification can be a powerful tool to study structure–function relationships in proteins. It is, however, necessary to determine whether there are gross structural changes that occur concomitantly with the chemical modification. This is of particular importance when there is more than a single residue modified. It is possible to make some independent assessment of the structural integrity of the protein after modification by various analytical methods, including size-exclusion chromatography, reverse-phase high-performance liquid chromatography, analytical ultracentrifugation, differential scanning calorimetry, and circular dichroism. The development of surface plasmon resonance technology (*see* Chapter “Epitope Mapping by Surface Plasmon Resonance”) is of value for the study of the kinetics of antigen–antibody reactions (33–41) and is useful for evaluating the effect of epitope modification on antigen–antibody interactions in a quantitative manner.

A list of reagents for chemical modification is given in **Table 1**.

Table 1
Reagents for the chemical modification of proteins

Reagent	Specificity/Conditions ^a	MW	References
Acetic anhydride	Lysine, α -amino groups, tyrosine hydroxyl; preferred reaction is at lysine; pH 8 or greater; reaction can be “driven” to α -amino groups at pH less than 6.5. Avoid nucleophilic buffers such as Tris; hydrolysis of the reagent is an issue above pH 9.5. Acetic anhydride has been used for trace labeling in the study of protein conformation, and more recently the deuterated derivative has been used in proteomics for differential isotope tagging.	102.1	(42–46)
<i>N</i> -Acetylimidazole (1-acetylimidazole)	Tyrosine hydroxyl groups, lysine ϵ -amino groups, transient reaction at histidine; neutral pH.	110.1	(47–51)
Bromoacetamide (2-bromoacetamide)	Cysteine, reaction with active site histidine residues, also reaction with lysine, methionine, and possibly carboxylic acids. Reaction at pH 5–9, but reaction with methionine at pH 3.0. Reaction rate below pH 7.5 is usually slow as the modification of cysteine requires thiolate anion (pK_a for cysteine is 8.7. Reaction is slower than iodoacetamide. A neutral reagent.	138	(52–56)
Bromoacetic acid (2-bromoacetic acid)	Reaction parameters similar to bromoacetamide except bromoacetic acid is a charged reagent at pH greater than 4 (pK_a is 2.7 at 25°C). Amide and acid derivatives can show different reaction patterns.	139	(57–61)
Bromoethylamine	Modification of sulfhydryl groups; conversion of cysteine to lysine analogue (<i>S</i> -2-aminoethylcysteine); reaction with cysteine at alkaline pH (see bromoacetamide). Reaction is reasonably specific for cysteine with possible modification at the amino-terminal α -amino group and histidine.	204.9 as HBr salt	(62–66)
<i>N</i> -Bromosuccinimide	Modification of tryptophan with some oxidative side reactions; pH 4–6.	178	(66–71)
2,3-Butanedione (diacetyl)	Modification of arginine residues; reversible reaction with the product stabilized by the presence of borate; reaction at alkaline pH.	86.1	(72–76)

Cyanate	Carbamylation of α -amino groups in proteins at alkaline pH with some preference toward the modification of N-terminal α -amino groups. Reaction also occurs at cysteine residues using 2-nitro-5-thiocyanatobenzoic acid.	NA	(77-81)
1,2-Cyclohexanedione	Modification of arginine in the presence of borate. At pH 7-9, the reaction is reversible; above pH 9, the reaction is irreversible with the formation of several products.	112.1	(82-86)
1,3-Dicyclohexyl-carbodiimide (DCC)	Modification of carboxyl groups in proteins (activates carboxyl groups which are then modified with a nucleophile such as glycine methyl ester; solubility issues have made EDC a more attractive reagent); also used for synthesis of phosphate ester bonds and peptide bonds; pH less than 5; modification requires protonated carboxyl group. The reagent has been used at more alkaline pH values. The modification of tyrosine and cysteine has been reported. DCC is an inhibitor of the proton-translocating ATPase in mitochondria and has been extensively used to characterize that activity.	206.3	(87-91)
Diethylpyrocarbonate	Modification of histidine residues in proteins with transient modification of tyrosine; possible reaction at amino groups. Disubstitution of histidine results in ring-opening.	162.1	(92-96)
1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC); <i>N</i> -(3-dimethylaminopropyl)- <i>N'</i> -ethylcarbodiimide	Modification of carboxyl groups in proteins frequently with <i>N</i> -hydroxysuccinimide. Used for zero-length cross-linking in proteins and for the coupling of proteins to matrices and for the preparation of protein conjugates.	155.2	(97-101)
<i>N</i> -Ethylmaleimide	Modification of sulfhydryl groups via Michael addition to the maleimide ring. There are some proteins that are distinguished by their modification with <i>N</i> -ethylmaleimide, including <i>N</i> -ethylmaleimide-sensitive fusion protein and soluble <i>N</i> -ethylmaleimide-sensitive factor attachment protein receptors.	125.1	(102-106)
2-Hydroxy-5-nitrobenzyl bromide (Koshland's reagent)	Modification of tryptophan by alkylation of the indole ring. Reaction at acid pH (pH 2-6). Disubstitution can occur. This was one of the first "reporter groups."	232.0	(107-111)
<i>N</i> -Hydroxysuccinimide	Functional group for modification of amino groups in proteins, frequently with carbodiimide for carboxyl modification and coupling in proteins.	115.1	(112-116)

(continued)

Table 1
(continued)

Reagent	Specificity/Conditions ^a	MW	References
2-Iminoethanol (Traut's reagent)	Insertion of a sulfhydryl group into a protein via modification of a lysine residue. This reaction can be used for other amino functions and can be useful for matrix building.	137.6 as HCl	(117–120)
Iodoacetamide	Reaction is faster than with bromo- or chloro derivatives. Reaction characteristics similar to bromoacetamide. As with bromoacetamide, iodoacetamide is neutral.	185	(121–126)
Iodoacetic acid	See bromoacetic acid for reaction conditions. As with bromoacetic acid, reagent is charged at pH greater than 5 (pK_a is 3.12 at 25°C).	186	(127–131)
Methyl acetimidate	Modification of amino groups. Imido esters are the functional groups for a number of cross-linking agents such as dimethylsuberimide. One of the more interesting imido esters is methyl picolinimide. Reaction at pH 8–10.	109.6 as HCl	(132–136)
Methyl methane thiosulfonate	Methyl methanethiosulfonate is one of a group of alkyl methanethiosulfonate derivatives which reversibly modify cysteine residues in proteins. Reaction occurs at slightly alkaline pH (pH 7.8).	126.2	(137–141)
2-Nitrophenylsulfenyl chloride (<i>o</i> -nitrophenyl-sulfenyl chloride)	Modification of tryptophan residues in proteins; reaction occurs at acid pH; modified tryptophan can be converted to the 2-thioltryptophan derivatives. This modification has been used to purify tryptophan peptides from protein hydrolyzates. An analogue, 2-(trifluoromethyl)-benzenesulfenyl chloride has been developed for use in mass spectrometry.	189.6	(142–146)
Phenylglyoxal	Modification of arginine residues in proteins; reaction accelerated in the presence of bicarbonate buffers; reaction at alkaline pH. <i>p</i> -Hydroxyphenylglyoxal and <i>p</i> -nitrophenylglyoxal are useful derivatives.	134.1 as hydrate	(146–151)
Sodium sulfite	Oxidative sulfiteolysis to cleave disulfide bonds; conversion of cysteine to <i>S</i> -sulfofocysteine.	126	(152–156)

Tetranitromethane	Nitration of tyrosine residues in proteins and possible cross-linking; also reacts with sulfhydryl groups; possible reaction with indole ring of tryptophan. Reaction at alkaline pH; does introduce a "reporter group" in proteins. The nitrotyrosine function can be reduced to aminotyrosine with sodium dithionite (sodium hydrosulfite). The modification with peroxynitrite is a similar reaction.	196	(157-161)
Trinitro-benzenesulfonic acid	Modification of amino groups in proteins; used for the determination of amino groups. Reaction at alkaline pH; also reacts with sulfhydryl groups.	293.2	(162-166)
Woodward's reagent K (<i>N</i> -ethyl-5-phenylisoxazolium-3-sulfonate)	Reagent used for the modification of carboxyl groups in proteins. Reaction at acidic pH.	253.3	(166-171)

^aAbsolute specificity of modification cannot be guaranteed. Conditions are most common with the caveat that specific buffer effects are observed

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