An overview on chemical modification of enzymes. The use of group-specific reagents

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Chemical modification is an important approach to the study of enzyme active sites. This article presents an overview of the methods used. The basic concepts, applications and limitations of chemical modification are outlined. The use of reagents specific for different amino acid side chains is also discussed.

Key terms: *chemical modification of enzymes; group-specific reagents.*

INTRODUCTION

Enzymes represent a most remarkable set of biomolecules, owing both to their high catalytic activity and their ligand specificity. One of the important goals of biochemistry is to learn how enzymes perform their task, which is fundamental for the very existence of life.

There are several ways of approaching the study of enzyme mechanisms. One of them is to analyze the kinetics of the reaction catalyzed by a certain enzyme. By this means one can learn about the order in which substrates bind and products leave the enzyme, and the velocity at which these processes take place. This analysis does not tell us, however, the detailed molecular mechanism by which these transformations occur, and how the structural elements of the enzyme contribute to them.

Enzymes and substrates come in close contact in a limited area of the enzyme surface called the 'active site', at which the catalytic process takes place. To learn about the catalytic mechanism of an enzyme it is, therefore, essential to study the structural elements of this site (amino acid side chains and prosthetic groups) and the three-dimensional conformation of the site.

The most powerful technique currently available for such a study is X-ray diffraction. If a diffraction pattern of sufficient resolution can be obtained, one can get a very accurate picture of the three-dimensional structure of the enzyme, and thus of its active site. This technique has been applied very successfully to many enzymes, some of the best known examples being lysozyme (Ford *et al*, 1974) and carboxypeptidase A (Quiocho and Lipscomb, 1971). With a good knowledge of the structural elements of the active site and their orientation, a reasonable mechanism for their participation in the catalytic process can be proposed.

The use of X-ray diffraction, however, suffers from some limitations. Among them is the need of obtaining the enzyme in a crystalline form adequate for analysis. The technique is also expensive, time-consuming and requires very specialized personnel and equipment. Besides, the question still remains as to how valid are results obtained in the crystalline state when extrapolated to aqueous solution (the condition under which enzymes operate). X-ray diffraction is discussed in greater detail in another article of this issue (Cid, 1996).

A host of other analytical techniques, individually less powerful than X-ray diffraction, is also being used to address structural questions. These techniques are important, not only as means of complementing or confirming X-ray data, but can also

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give information of their own. Very often they are the only ways of probing active-site structure and function. Among the physical method are nuclear magnetic resonance, mass spectroscopy and circular dicroism. Chemical methods include the combination of monoclonal antibodies and partial proteolysis, site-directed mutagenesis and chemical modification. Several of these techniques are discussed in this and subsequent articles.

CHEMICAL MODIFICATION - GENERAL ASPECTS

Of special importance in probing active-site structure is chemical modification. A reagent is placed in contact with the enzyme and a chemical reaction occurs. This reagent will bind covalently to amino acid side-chains in the enzyme and will produce changes in some measurable property (or properties) of the enzyme. Ideally, the reagent should be of sufficient selectivity to combine with only one residue, and cause minimal alterations in the conformation of the enzyme. This covalent derivatization is then correlated to the enzyme property under consideration, so that a function can be suggested for the modified residue.

Of the 20 natural amino acids, only those possessing a polar side-chain are normally the object of chemical modification. The chemical reactivity of these groups is basically a function of their nucleophilicity. This nucleophilicity can be influenced in turn by several factors which, as will be discussed below, can greatly affect the outcome of chemical modification.

Two main types of chemical modification can be recognized: one is based on the use of group-specific reagents, and the second utilizes affinity labels.

The modifiers belonging to the first class must be sufficiently reactive to bind covalently with an amino acid side-chain, and at the same time show high specificity to react (ideally) with only one type of side-chain. Many reagents for the modification of different amino acid side-chains have been developed and utilized, so that a large battery of compounds is available (many of them commercially).

The principal problem encountered with this type of reagent is lack of specificity. In addition, more than one residue of a certain amino acid is normally present in an enzyme molecule, making it difficult to achieve selectivity towards one single residue.

To overcome these limitations, researchers doing chemical modification increasingly turn to affinity labels. These compounds are chemically reactive analogs of enzyme ligands. Owing to this structural similarity, they show affinity for the ligand binding site, and therefore bind selectively on the enzyme surface. By means of their reactive group, these analogs can form a covalent bond with the enzyme. Since the specificity of these reagents is given by their affinity for a binding site, it is less important, and may even be desirable, for them not no react specifically with only one type of amino acid side-chain. These reagents show saturation kinetics and compete with their natural ligand counterpart for the binding site on the enzyme. The only important limitation of these reagents is that they must be prepared ad hoc for each enzyme or group of enzymes.

Two important subsets of affinity labels are the photoaffinity labels and the suicide inhibitors. Photoaffinity labels are activated by irradiation with light, and thus their reactivity can be closely controlled. The active species are free radicals which readily react with almost any chemical group in their neighborhood. These labels may also combine with non-polar side-chains. Suicide inhibitors are non-reactive compounds which act as substrates. By virtue of their transformation into products, a reactive group is generated, so that they readily bind to side-chains located in their vicinity at the active site. By their very nature, suicide inhibitors can be powerful pharmacological agents in the treatment of certain diseases.

The properties of group-specific reagents are discussed in more detail below. Affinity labels and photoaffinity labels are the subject of later chapters in this volume (see Bazaes, 1996; Schäfer & Schuhen, 1996). The reader interested in suicide inhibitors is referred to the review by Walsh (1984).

PROBLEMS AND LIMITATIONS IN CHEMICAL MODIFICATION

The investigator doing chemical modification should be aware of several limitations inherent in this technique. They have been summarized by Cohen (1970), and are reviewed in the following paragraphs.

Few, if any, chemical modifiers are absolutely specific for a certain amino acid side-chain This specificity can be influenced by the experimental conditions used, principally by the pH.

Absolute selectivity for one single amino acid residue is very seldom achieved. One particular enzyme may possess many residues of a certain kind, which can potentially react with a group-specific reagent. The reactivity of an amino acid side-chain is greatly influenced, however, by its microenvironment. At the active site of enzymes, which has a less polar environment, amino acid residues often show a markedly different pKa than the free amino acid. A good example is found in the enzyme oxaloacetate decarboxylase, possessing a lysine of pKa = 5.9 (Schmidt and Westheimer, 1971). These amino acids are often much more reactive, thus increasing the selectivity with which they may be labeled by group-specific reagents.

It is unlikely that any chemical modification can be accomplished without some change in the conformation of the protein. If conformational changes occur, they may be responsible for any observed change in the biological property being considered. Few investigators actually monitor conformational changes, although only minor changes have been observed in some cases studied (Kirschner and Schachman, 1973; Horiike *et al*, 1979). The use of reagents of small size may help reduce conformational changes.

The behavior of a reagent towards a free amino acid is only a partial indication on how it will react with the side-chain of the same amino acid in a protein, and the behavior towards one protein is only a partial indication of its behavior towards others. As indicated above, the microenvironment around a specific residue is a key factor in determining its reactivity. Several factors influence this microenvironment. One is polarity, which affects the pKa of the dissociable side-chains. Other important factors are hydrogen bonding effects, which may stabilize a neutral or ionic species, electrostatic effects (presence of charges in the vicinity of the group under study), and steric effects by other side-chains, which may hinder the approach of the reagent (Cohen, 1970).

All the experimental variables can have an effect on the result of the modification. Temperature may be important if one is following the kinetics of modification. pH is another essential variable, since it influences the degree of ionization of both the reagent and the group under study. The nature of the buffer used is at times important. For example, more effective modification of arginines is obtained in borate than other buffers when butanedione is used as reagent, since borate stabilizes the reaction products (Riordan, 1979).

TYPES OF INFORMATION OBTAINABLE FROM CHEMICAL MODIFICATION STUDIES

Stoichiometry of modification

It is very important to determine the number of molecules of modifier incorporated per active unit of enzyme in order to establish if non-specific modification has occurred. There are several means of accomplishing this, the choice depending on the type of reagent used. On occasions, the formation of the covalent bond between modifier and target residue produces a change in the absorption spectrum of the modifier. This may be a simple way of quantifying the modification, although a knowledge of the extinction coefficient of the complex is essential; this value, however, may vary from enzyme to enzyme, as shown for the modification of lysine residues by pyridoxal phosphate (Bazaes *et al*, 1980).

Amino acid analysis of the modified enzyme can give clues as to the type and number of residues modified; the precision of this technique is of the order of 3-5%, so an accurate measurement of the disappearance of a particular residue may be difficult if the protein under study has a large content of this residue (Rohrbach and

Bodley, 1977). The appearance of a new product, such as S-carboxymethylcysteine in the modification of cysteine residues by iodoacetate, may be more easily detected.

The most common means of stoichiometry determination is to follow the incorporation of a radiolabeled modifier. It is more difficult, however, with this technique to determine the degree of non-specific modification.

Correlation of the degree of modification with change in a biological property (most commonly, enzyme activity)

A correlation of modification with alteration of a biological property is essential if one wants to ascribe a function to the modified residue. The simplest and ideal case is a linear relationship between these two variables. Usually such a correlation (or lack thereof) is established by means of a plot of residual activity against number of residues modified. The data can be extrapolated to zero residual activity to obtain the number of essential residues. If a linear response is obtained, one expects the modification to be an 'all or none' process, so that the total population of enzyme molecules is composed of a set of native and another of totally inactive molecules. This can be confirmed by determining the kinetic constants (K_m and K_{cat}) of a partially modified sample of the enzyme. Horiike and McCormick (1979) have analyzed the validity of this approach and have pointed out that the extrapolation is valid if any non-essential residue reacts at least 100 times slower than the essential one.

If the above plot gives a non-linear result, the number of essential residues can still be established by means of the statistical analysis of Tsou (1962). This method is based on a plot of the total number of modified residues against the residual activity raised to the reciprocal power of a small integer (i = 1, 2, 3, etc.). Several plots are made of the experimental data, each with a different value for i, and the resulting curves are analyzed statistically for the best fit to a straight line. The value of i corresponding to the best fit is the number of essential residues. Non-essential residues must react either much faster or much slower for the method to be valid. The reader is referred to Paterson and Knowles (1972) and Lundblad (1994) for a detailed description and applications of this interesting method.

Another method used to detect the identity and number of essential amino acid residues is the kinetic approach of Ray and Koshland (1961). This method is based on obtaining a relationship between the rate of loss of biological activity and the rate of modification of amino acid residues. To establish this relationship, the pseudo first-order rate constants of these processes must be determined. Limitations of this approach include the requirement of pseudo first-order kinetics (not always met in chemical modification), and the use of experimental conditions such that the velocity of modification can be accurately followed to allow a precise estimation of the constants.

Kinetic analysis of chemical modification

Such an analysis can give information on the kinetic mechanism of the inactivation process. It can also be used to determine the dissociation constant of a ligand-enzyme complex and of the pKa's of the reactive groups. The second application can be particularly useful when more conventional methods such as equilibrium dialysis cannot be employed, especially if the affinity for the ligand is low ($K_d > 10^{-3}$ M). All these methods require, however, that the inactivation process follows pseudo first-order kinetics. For more detail, see the article by Cardemil (1996) in this volume.

Sequencing of a peptide containing the modified amino acid

The labeled protein can be subjected to chemical or proteolytic cleavage followed by separation of the labeled peptide. HPLC has proved particularly useful in this respect (Hunkapiller *et al*, 1984). Sequencing of the peptide can give information on the location of the essential residue in the protein primary structure. An example is the labeling and sequencing of a peptide from bovine muscle pyruvate kinase containing an essential lysine residue (Johnson *et al*, 1979).

Assignment of possible functions to the modified amino acid residue

Essential amino acid residues in the enzyme active site can participate in substrate binding or in catalysis. It is very difficult to assign these specific functions to a residue on the basis of chemical modification alone. However, certain types of experiments can give valuable information on the essential nature of a residue. Among them are protection experiments, in which the effect of substrate or other ligands on the velocity and the degree of modification is studied. The enzyme fructose-6-phosphate 2 kinase - fructose 2,6 bisphosphatase has been modified by pyridoxal 5' phosphate. Protection of the kinase activity is observed when modifying in the presence of fructose 2,6 bisphosphate and to a lesser extent of ATP. Two lysines are modified in the unprotected enzyme, while protection drastically reduces the modification, strongly suggesting that these residues are important for enzyme activity (Kitajima *et al*, 1985).

Sometimes a combination of chemical modification kinetics and substrate-catalyzed kinetics can reveal particularly useful mechanistic information. For example, this approach has provided evidence that catalytic competence in cysteine proteinases requires some structural change in addition to the formation of a thiolate$^-$/imidazolium$^+$ ion pair (Willenbrock and Brocklehurst, 1984).

Correlation of the reversal of the modification with recovery of the altered biological function is additional useful evidence for establishing the possible function of a residue. Bazaes *et al* (1980) have shown that the modification of the only cysteine residue in liver phosphomevalonate kinase produces total loss in activity; this activity is almost fully recovered if the modification is reversed by dithiothreitol, thus supporting a possible essential role for this residue at the active site.

To confirm the function assigned to a modified residue, it is very important to determine if the alteration caused by the modification is due to a steric effect. This question can be approached by transforming or changing the putative essential residue into another amino acid of similar size and general properties. One, although limited, way to accomplished this is by 'chemical mutation'. An example is the conversion of an essential cysteine of papain into serine, which resulted in a total loss of enzyme activity (Clark and Lowe, 1978). More important and of a much broader application is "site-specific mutagenesis". This technique consists in the 'in vitro' replacement of a specific purine or pyrimidine base in the protein's gene sequence so as to obtain a different amino acid in the designated position. This powerful technique, introduced by Fersht in 1982, is discussed in detail in a later article by Moreno-Hagelsieb and Soberón (1996).

Site-directed mutagenesis is often used to confirm results obtained by chemical modification. Glycinamide ribonucleotide transformylase is inactivated by the affinity reagent N^{10}-(bromoacetyl)-5,8-dideazafolate which binds to Asp 144. Replacement of this residue by asparagine by mutagenesis results in a catalytically inactive enzyme (Inglese *et al*, 1990). An interesting example where mutagenesis does not confirm the results of chemical modification has been observed with tryptophan synthase. Chemical modification studies of the sulfhydryl groups of the enzyme α subunit suggested that they play important catalytic roles; however, replacement of Cys 81 or Cys 118 by serine gave fully active mutants (Ahmed *et al*, 1988). The chemical modification experiments had been performed with N-ethylmaleimide; it is likely that the observed inactivation was due to steric hindrance caused by the bulky side-chains of the inhibitor.

Both techniques can also complement each other when analyzing the catalytic function of an amino acid residue. For instance, Kim *et al* (1994) substituted the pyridoxal 5'-phosphate-binding lysine of a thermostable aspartate aminotransferase by cysteine, leading to a loss in activity. Further, they modified this residue by chemical modification obtaining several different lysine sulfur analogs, and thereby partially recovering the wild-type activity.

Study of allosteric and cooperative properties

Although chemical modification of enzymes is often associated with alterations at the enzyme active site, it has also been used successfully to analyze essential residues at allosteric sites. Kemp discusses this point elsewhere in this volume. Slebe *et al* (1983) have studied by chemical modification the existence of residues essential for cooperative interactions in fructose 1,6 bisphosphatase. These authors show that loss in cooperativity by selective carbamylation of the AMP binding sites of this enzyme can be correlated with the modification of a lysine residue in each subunit.

Location of amino acid residues in the tertiary structure of a protein

The concepts of 'exposed' and 'buried' residues have been proposed to account for the degree of accessibility of amino acid residues to chemical modification. The 'exposed' residues are assumed to be located on the surface of the protein, while the 'buried' ones are hidden from the reagent in its hydrophobic interior. The 'buried' residues should become accessible as a result of denaturation of the enzyme. This topic is discussed further by Silva (1996) in this volume.

Crosslinking by means of chemical modification

Reagents that contain two reactive functional groups can be used to form intramolecular or intermolecular crosslinks between amino acid residues of proteins. This strategy is useful in establishing distances between the reactive side-chains, and can thus give insight into the tertiary or quaternary structure of a protein. A variety of reagents have been utilized for this purpose, among them 1,3 dibromoacetate, glutaraldehyde, dimethyl suberimidate, etc. A detailed account of these reagents and a survey of their application to the study of many proteins can be found in Lunblad (1994). Photoaffinity crosslinking is discussed by Schäfer and Schuhen (1996) in this volume.

Alteration of enzyme specificity

Chemical modification of enzymes can change not only their catalytic parameters, but also their catalytic behavior in a qualitative manner (Kaiser *et al*, 1985). An interesting example is that of the flavopapains, where the hydrolytic activity of papain is transformed into the oxido-reductase activity of a flavoprotein by alkylation of an active-site cysteine residue with a flavine derivative (Kaiser and Lawrence, 1984).

SOME IMPORTANT REAGENTS USED IN THE MODIFICATION OF INDIVIDUAL AMINO ACID RESIDUES

As indicated above, polar amino acid residues which can show nucleophilicity at a certain pH range, can be subjected to chemical modification by group-specific reagents. These include the acidic amino acids glutamate and aspartate, the basic amino acids lysine, arginine and histidine, the polar uncharged amino acids serine, cysteine and tyrosine, and the side-chains of methionine (possessing a nucleophilic sulfur) and tryptophan (heterocyclic indole side-chain). Numerous reagents have been introduced for the modification of these residues. A detailed review of them is beyond the scope of this article, so only a selected group is discussed in some detail. A good general review is found in Glazer (1976); Lundblad (1994) gives a comprehensive and updated account of group-specific reagents, and Glazer *et al* (1975) discuss experimental aspects in the use of many of these reagents.

Acid amino acids (Glu and Asp)

The carboxylic side-chain of these amino acids has been modified by a number of different reagents. Hoare and Koshland (1966) introduced the use of water-soluble carbodiimides as catalysts for the specific modification of carboxyl groups in proteins by amines. This is the most widely used method. The binding of amines such as [^{14}C] glycine ethyl ester can be detected by the appearance of a new peak in amino acid analysis. Some reactivity by serine, cysteine, and tyrosine under certain experimental

conditions has been reported (Carraway and Koshland, 1972).

The conversion of carboxyl groups to esters by means of trialkyl oxonium fluoroborate salts has been used by several authors, among them Paterson and Knowles (1972). These investigators modified pepsin with [^{14}C] trimethyloxonium fluoroborate, establishing that at least two carboxyl groups are essential for the activity of the enzyme. Some reactivity is observed with methionine and histidine (Yonemitsu *et al*, 1969).

Isoxasolium salts were introduced by Woodward *et al*, 1961). Several of these derivatives have been used for chemical modification, especially N-ethyl-5-phenyl-isoxazolium-3'-sulfonate (Woodward's reagent K). Arana and Vallejos (1981) have used this reagent to study essential carboxyl groups in chloroplast coupling factor, comparing its effect with that of a carbodiimide. Sinha and Brewer (1985) described a method to quantify the degree of modification based on the absorbance of the adduct at 340 nm. This method has been applied, for instance, by Komissarov *et al* (1995) who have found a reactive aspartate in position 5 of uridine phosphorylase. Llamas *et al* (1986) have studied the reaction of Woodward's reagent K with a series of model nucleophiles. They have shown that stable adducts with absorbance at 320-350 nm can be formed by compounds other than carboxyl groups, thus questioning the selectivity of the reagent. To allow differentiation, they suggest the reaction of the product with hydroxylamine, which in the case of carboxyls should yield stable hydroxamates.

Arginine

This amino acid possesses a highly basic guanidino group; it has been proposed that it plays an essential role in the active site of enzymes binding anionic substrates (Riordan, 1979). Most of the chemical modification studies of arginine residues have been done with dicarbonyl reagents, two of which, butanedione and phenylglyoxal, will be discussed further.

Butanedione was introduced by Yankeelov (1972), and was made popular as an arginine modifier by Riordan. He observed that borate buffer stabilizes the arginine-butanedione complex, although the reaction is reversible (Riordan, 1979). It has been pointed out that the reaction must be performed in the dark, since butanedione may act as a photosensitizing agent and cause the destruction of other residues, particularly tryptophan, histidine and tyrosine (Fliss and Viswanatha, 1979; Gripon and Hofmann, 1981). One of the many enzymes studied with this reagent is muscle pyruvate kinase (Cardemil and Eyzaguirre, 1979); this enzyme is reversibly inactivated with concomitant loss in arginine as determined by amino acid analysis; protection experiments suggest that an essential arginine residue is located near the phosphate binding site of the substrate phosphoenolpyruvate.

Phenylglyoxal was first used by Takahashi (1968) as an arginine-modifying reagent. Two molecules of phenylglyoxal normally bind per arginine residue, and the reaction is irreversible. Reactivity with alpha-amino groups of peptides has been described (Takahashi, 1968). Since [^{14}C]-phenylglyoxal can be easily synthesized (Schloss *et al*, 1978), its incorporation into proteins can readily be followed. Communi *et al* (1995) have studied the inactivation of inositol 1,4,5-trisphosphate 3-kinase A by phenylglyoxal, and using the [^{14}C]-labeled compound have found a reactive arginine at position 317 of the enzyme. Cheung and Fonda (1979) have shown that the reaction of phenylglyoxal with arginine in model compounds is faster in bicarbonate buffer, while α-amino groups appear unreactive under the same conditions.

Lysine

The epsilon amino group of this amino acid in its unprotonated form is a very reactive nucleophile in proteins. The pKa of this group is usually around 10. However, Lys residues of lower pKa can be found in enzymes as a result of microenvironmental effects; they are considerably more reactive and can therefore be selectively modified. A value of 5.9 has been found for a reactive lysine residue in oxaloacetate decarboxylase (Schmidt and Westheimer, 1971).

Many compounds have been utilized for lysine modification. A useful reagent is trinitrobenzenesulfonate (TNBS). The reaction with amino groups causes specific spectral changes which can be followed at 420 or 367 nm (Lundblad, 1994). A highly reactive lysine residue of bovine muscle pyruvate kinase has been labeled with TNBS and a tryptic peptide containing the modified residue has been isolated and sequenced (Johnson *et al*, 1979).

Carbamylation of amino acids by means of cyanate has been used extensively. One clear advantage of this reagent is the small size of the cyanate ion. Reaction is also observed with histidines and cysteines, forming unstable carbamyl derivatives; carbamylation of the active-site serine of chymotrypsin (with concomitant loss of activity) has been reported (Shaw *et al*, 1964). Practical uses of this reagent are discussed by Stark (1972).

Pyridoxal phosphate (PLP), a natural derivative of vitamin B_6, is a very specific modifier of amino groups. The reaction leads to the reversible formation of a Schiff's base which can be stabilized by reduction with sodium borohydride. The stoichiometry of incorporation can be followed either spectrophotometrically (the reduced pyridoxyl derivative absorbs with a maximum at 325 nm; Glazer *et al*, 1975, page 131) or by reduction with ^{3}H-borohydride. A typical example of the use of this reagent in the determination of essential lysine residues is the work of Schnackerz and Noltmann (1971) on phosphoglucose isomerase. The authors find a very specific reaction with the incorporation of one molecule of PLP per subunit of enzyme.

Histidine

The role of histidine in enzyme-active sites has been reviewed by Schneider (1978). Two main procedures are used for the modification of the imidazole ring of histidine. Photo-oxidation was the first method introduced. Unfortunately, photo-oxidation shows low specificity, since methionine, tryptophan and to a lesser extent tyrosine, serine and threonine are also modified (Lundblad, 1994). Methylene blue and Rose Bengal are two of the commonly used dyes in this procedure.

Diethylpyrocarbonate is the most commonly used reagent for histidine modification (Miles, 1977). This reagent shows good specificity at near-neutral pH. The reaction leads to an increase in absorbance at 240 nm, and results in the substitution (carboxyethylation) of one of the imidazole nitrogens. The substitution can be reversed at alkaline pH, resulting in recovery of histidine. The reagent is somewhat unstable in aqueous media, especially at higher pH. Horiike *et al* (1979) have used this reagent with pyridoxamine (pyridoxine)-5'-phosphate oxidase, and have shown that the enzyme is inactivated with the modification of a crucial His residue, without noticeable conformational perturbations. Dominici *et al* (1985) have shown that diethylpyrocarbonate modifies one His residue of 3,4 dihidroxyphenylalanine decarboxylase which is essential for activity.

Cysteine

Owing to the high nucleophilicity of the thiol group (especially the thiolate anion), cysteine is the most reactive amino acid residue in proteins, and therefore can be modified by a great number of reagent. A detailed review of the reactions of thiol groups in proteins can be found in the chapter by Liu (1977).

Alkylating agents, especially iodoacetate and iodoacetamide, represent one of the most important groups of compounds used in thiol modification. Iodoacetate is customarily used to carboxymethylate sulfhydryl groups of proteins prior to amino acid analysis or sequencing (Crestfield *et al*, 1963); this treatment prevents Cys degradation by formation of a stable carboxymethyl cysteine which can be easily identified by amino acid analysis. Other haloacids or amides such as bromoacetic acid can be used, but the reaction is slower than with the iodo derivatives, because iodide is a better leaving group. Imidazole groups, although considerably less reactive, may also combine with haloacetic acids; an example of high imidazole reactivity is found in ribonuclease (Heinrikson *et al*, 1965).

N-ethylmaleimide has been a valuable reagent for sulfhydryl group modification. The reaction shows good specificity, and can be followed spectrophotometrically. The ethyl group can be replaced by a spin label, and this has been used effectively to study the mobility of the arm in the pyruvate dehydrogenase complex (Ambrose and Perham, 1976).

Ellman (1959) introduced 5,5-dithiobis-(2-nitrobenzoic) acid (DTNB), which forms mixed disulfides when reacting with SH groups, liberating 2-nitro-5-thiobenzoic acid. Since the thiolate formed has a strong absorbance at 410 nm, the reaction can be easily followed spectrophotometrically. An example of the use of this reagent is the study of an essential cysteinyl residue in phosphomevalonate kinase (Bazaes *et al*, 1980). Applications of the more versatile disulfides containing the 2-mercaptopyridine group have been reviewed by Brocklehurst (1982).

Oxidation of thiol groups, other than by thiol-disulfide interchange, is another obvious chemical modification that would be expected to provide a high level of specificity. A particularly convenient chromophoric oxidizing agent for thiol groups is benzofurozan (see Salih and Brocklehurst, 1983).

Organic mercurials have been one of the oldest reagent used for cysteine modification, the most common one being p-chloromercuribenzoate. This compound becomes the hydroxy derivative when dissolved in water, and shows a strong increase in absorbance at 255 nm upon reacting with thiols. Bai and Hayashi (1979) have studied the effect of this compound on the activity of carboxypeptidase Y towards several substrates.

Tyrosine

Tetranitromethane has been a widely used reagent in tyrosine modification studies because of its high specificity and reactivity under mild conditions. This compound nitrates Tyr, producing an ionizable chromophore, 3-nitrotyrosine. Riordan *et al* (1967) have explored the topology of the active site of nitrated carboxypeptidase A (which retains activity) following perturbations in the nitrotyrosyl spectrum through the binding of substrates and inhibitors.

Serine

This residue has been subjected to relatively few studies by means of group-specific chemical modification. A well-known case of participation of serine residues in enzyme active sites is in the so-called serine proteases, where a Ser residue shows high reactivity towards acylating reagents such as diisopropylfluorophosphate (Kraut, 1977).

Methionine

This amino acid residue, although of low polarity, can be subjected to chemical modification owing to the nucleophilicity of the thioether sulfur. It is difficult to achieve selectivity when modifying this residue under mild conditions, but methionine can be oxidized to methionine sulfoxide by means of several reagents (Lundblad, 1994). Alkylation of this residue has also been reported, using for example, iodoacetate (Gundlach *et al*, 1959).

Tryptophan

The indole ring of tryptophan can be subjected to chemical modification. A commonly used reagent is N-bromosuccinimide (NBS), which oxidizes the indole residue to an oxindole derivative. The reaction can be monitored by following the decrease in absorbance at 280 nm. Reaction with tyrosine residues can occur, and this can interfere with the spectral measurements (Ohnishi *et al*, 1980). Warwick *et al* (1972) have studied the effect of NBS on dihydrofolate reductase; they observed an initial increase in activity due to oxidation of a cysteinyl residue followed, at higher reagent concentrations, by a loss in enzymatic activity associated with the oxidation of one Trp residue.

Koshland and co-workers have introduced the reagent 2-hydroxy-5-nitrobenzyl bromide (Horton and Koshland, 1965). This reagent forms a hydroxynitrobenzyl derivative, which shows spectral properties that are

sensitive to changes in the microenvironment (Lundblad, 1994).

CONCLUSION

This article is intended to serve as an overview of the methods used in the chemical modification of enzymes. The basic concepts, applications and limitations of chemical modification have been outlined. This technique, coupled with additional approaches, can help in the understanding of the structure and function of enzymes in solution.

REFERENCES

AHMED SA, KAWASAKI H, BAUERLE R, MORITA H, MILES EW (1988) Site-directed mutagenesis of the α subunit of tryptophan synthase from *Salmonella typhimurium*. Biochem Biophys Res Commun 151: 672-678

AMBROSE MC, PERHAM RN (1976) Spin-label study of the mobility of the enzyme-bound lipoic acid in the pyruvate dehydrogenase multienzyme complex of *Escherichia coli*. Biochem J 155: 429-432

ARANA JL, VALLEJOS RH (1981) Two different types of essential carboxyl groups in chloroplast coupling factor. FEBS Lett 123: 103-106

BAI Y, HAYASHI R (1979) Properties of the single sulfhydryl group of carboxypeptidase Y. J Biol Chem 254: 8473-8379

BAZAES SE (1996) Affinity labels as probes for the study of the nucleotide binding site of enzymes. Biol Res 29: 21-30.

BAZAES S, BEYTIA E, JABALQUINTO AM, SOLIS DE OVANDO F, GOMEZ I, EYZAGUIRRE J (1980) Pig liver phosphomevalonate kinase. 2. Participation of cysteinyl and lysyl groups in catalysis. Biochemistry 19: 2305-2310

BROCKLEHURST K (1982) Two-protonic-state electrophiles as probes of enzyme mechanism. Meth Enzymol 87: 427-469

CARDEMIL E (1996) Chemical modification of enzymes: Kinetics aspects. Biol Res 29: 13-20.

CARDEMIL E, EYZAGUIRRE J (1979) Evidence of essential arginyl residues in rabbit muscle pyruvate kinase. Arch Biochem Biophys 192: 533-538

CARRAWAY KL, KOSHLAND DE Jr (1972) Carbodiimide modification of proteins. Meth Enzymol 25: 616-623

CHEUNG S-T, FONDA ML (1979) Reaction of phenylglyoxal with arginine. The effect of buffers and pH. Biochem Biophys Res Commun 90: 940-947

CID H (1996) Active-site studies of enzymes by X-ray diffraction methods. Biol Res 29: 101-126

CLARK PI, LOWE G (1978) Conversion of the active-site cysteine residue of papain into a dehydro-serine, a serine and a glycine residue. Eur J Biochem 84: 293-299

COHEN LA (1970) Chemical modification as a probe of structure and function. The Enzymes, 3rd ed. 1: 147-211

COMMUNI D, LECOCQ R, VANWEYENBERG V, ERNEUX C (1995) Active site labelling of inositol 1,4,5-trisphosphate 3-kinase A by phenylglyoxal. Biochem J 310: 109-115

CRESTFIELD AM, MOORE S, STEIN WH (1963) The preparation and enzymatic hydrolysis of reduced and S-carboxymethylated proteins. J Biol Chem 238: 622-627

DOMINICI P, TANCINI B, VOLTATTORNI CB (1985) Chemical modification of pig kidney 3,4 dihydroxyphenylalanine decarboxylase with diethyl pyrocarbonate. J Biol Chem 260: 10583-10589

ELLMAN GL (1959) Tissue sulfhydryl groups. Arch Biochem Biophys 82: 70-77

FLISS H, VISWANATHA T (1979) 2,3 butanedione as a photosensitizing agent: application to α-amino acids and α-chymotrypsin. Can J Biochem 57: 1267-1272

FORD LO, JOHNSON LN, MACHIN PA, PHILLIPS DC, TJIAN R (1974) Crystal structure of a lysozyme-tetrasaccharide lactone complex. J Mol Biol 88: 349-371

GLAZER AN (1976) The chemical modification of proteins by group-specific and site-specific reagents. The Proteins, 3rd ed. 2: 1-103

GLAZER AN, DELANGE RJ, SIGMAN DS (1975) Chemical modification of proteins. In: WORKS TS, WORK E (eds) Laboratory Techniques in Biochemistry and Molecular Biology. Vol 4, part 1. Amsterdam: North-Holland/Elsevier. pp 167-179

GUNDLACH HG, MOORE S, STEIN WH (1959) The reaction of iodoacetate with methionine. J Biol Chem 234: 1761-1764

GRIPON J-C, HOFMANN T (1981) Inactivation of aspartyl proteinases by butane-2,3-dione. Biochem J 193: 55-65

HEINRIKSON RL, STEIN WH, CRESTFIELD AM, MOORE S (1965) The reactivities of the histidine residues at the active site of ribonuclease toward halo acids of different structures. J Biol Chem 240: 2921-2934

HOARE DG, KOSHLAND DE Jr (1966) A procedure for the selective modification of carboxyl groups in proteins. J Am Chem Soc 88: 2057-2058

HORIIKE K, MCCORMICK DB (1979) Correlations between biological activity and the number of functional groups chemically modified. J Theoret Biol 79: 403-414

HORIIKE K, TSUGE H, MCCORMICK DB (1979) Evidence for an essential histidyl residue at the active site of pyridoxamine (pyridoxine)-5'phosphate oxidase from rabbit liver. J Biol Chem 254: 6638-6643

HORTON HR, KOSHLAND DE Jr (1965) A highly reactive colored reagent with selectivity for the tryptophanyl residue in proteins, 2 Hydroxy-5-nitrobenzyl bromide. J Am Chem Soc 87: 1126-1132

HUNKAPILLER MV, STRICKLER JE, WILSON KJ (1984) Contemporary methodology for protein structure determination. Science 226: 304-311

INGLESE J, SMITH JM, BENKOVIC, SJ (1990) Active-site mapping and site-specific mutagenesis of glycinamide ribonucleotide transformylase from *Escherichia coli*. Biochemistry 29: 6678-6687

JOHNSON SC, BAILEY T, BECKER RR, CARDENAS JM (1979) Isolation and sequence determination of a peptide located in or near the active site of bovine muscle pyruvate kinase. Biochem Biophys Res Commun 90: 525-530

KAISER ET, LAWRENCE DC (1984) Chemical mutation of enzyme active sites. Science 226: 505-511

KAISER ET, LAWRENCE DS, ROKITA SE (1985) The chemical modification of enzymatic specificity. Annu Rev Biochem 54: 565-595

KIM D-W, YOSHIMURA T, ESAKI N, SATOH E, SODA K (1994) Studies of the active-site lysyl residue of

thermostable aspartate aminotransferase: combination of site-directed mutagenesis and chemical modification. J Biochem 115: 93-97

KIRSCHNER MW, SCHACHMAN HK (1973) Conformational studies of the nitrated catalytic subunit of aspartate transcarbamylase. Biochemistry 12: 2987-2997

KITAJIMA S, THOMAS H, UYEDA K (1985) Effect of modification of lysine residues of fructose 6-phosphate 2-kinase: fructose-2,6 bisphosphatase with pyridoxal 5' phosphate. J Biol Chem 260: 13995-14002

KOMMISSAROV A, ROMANOVA DV, DEBABOV VG (1995) Complete inactivation of *Escherichia coli* uridine phosphorylase by modification of Asp^5 with Woodward's reagent K. J Biol Chem 270: 10050-10055

KRAUT J (1977) Serine proteases: structure and mechanism of catalysis. Annu Rev Biochem 46: 331-358

LIU T-Y (1977) The role of sulfur in proteins. The Proteins, 3rd ed. 3: 239-402

LLAMAS K, OWENS M, BLAKELEY RL, ZERNER B (1986) N-Ethyl-5-phenylisoxazolium-3'-sulfonate (Woodward's Reagent K) as a reagent for nucleophilic side chains of proteins. J Am Chem Soc 108: 5543-5548

LUNDBLAD RL, (1994) Techniques in Protein Modification. Boca Raton, FL: CRC Press

MILES EW (1977) Modification of histidyl residues in proteins by diethylpyrocarbonate. Meth Enzymol 47: 431-442

MORENO-HAGELSIEB G, SOBERON X (1996) Protein enginering as a powerful tool for the chemical modification of enzymes. Biol Res 29: 127-140

OHNISHI M, KAWAGISHI T, ABE T, HIROMI K (1980) Stopped-flow studies on the chemical modification with N-bromosuccinimide of model compounds of tryptophan residues. J Biochem 87: 273-279

PATERSON AK, KNOWLES JR (1972) The number of catalytically essential carboxyl groups in pepsin. Modification of the enzyme by trimethyloxonium fluoroborate. Eur J Biochem 31: 510-517

QUIOCHO FA, LIPSCOMB WN (1971) Carboxypeptidase A: a protein and an enzyme. Adv Prot Chem 25: 1-78

RAY WJ Jr, KOSHLAND DE Jr (1961) A method for characterizing the type and numbers of groups involved in enzyme action. J Biol Chem 236: 1973-1979

RIORDAN JF (1979) Arginyl residues and anion binding sites in proteins. Mol Cell Biochem 26: 71-92

RIORDAN JF, SOKOLOVSKY M, VALLEE BL (1967) Environmentally sensitive tyrosyl residues. Nitration with tetranitromethane. Biochemistry 6: 358-361

ROHRBACH MS, BODLEY JW (1977) Selective chemical modification of Escherichia coli elongation factor G: butanedione modification of an arginine essential for nucleotide binding. Biochemistry 16: 1360-1363

SALIH E, BROCKLEHURST K (1983) Investigation of the catalytic site of actinidin by using benzofuroxan as a reactivity probe with selectivity for the thiolate-imidazolium ion pair system of cysteine proteinases. Biochem J 213: 713-718

SCHÄFER H-J, SCHUHEN A (1996) Photoaffinity labeling and photoaffinity crosslinking of enzymes. Biol Res 29: 31-46

SCHLOSS JV, NORTON IL, STRINGER CD, HARTMAN FC (1978) Inactivation of ribulosebisphosphate carboxylase by modification of arginyl residues with phenylglyoxal. Biochemistry 17: 5626-5631

SCHMIDT DE, WESTHEIMER FH (1971) pK of the lysine amino group at the active site of acetoacetate decarboxylase. Biochemistry 10: 1249-1253

SCHNACKERZ KD, NOLTMANN EA (1971) Pyridoxal 5' phosphate as a site-specific protein reagent for a catalytically critical lysine residue in rabbit muscle phosphoglucose isomerase. Biochemistry 10: 4837-4843

SCHNEIDER F (1978) Histidine in enzyme active centers. Angewandte Chemie (Intl edn) 17: 583-592

SHAW DC, STEIN WH, MOORE S (1964) Inactivation of chymotrypsin by cyanate. J Biol Chem 239: PC671-673

SILVA E (1996) Sensitized photo-oxidation of amino acids in proteins: Some important biological implications. Biol. Res 29: 57-67

SINHA U, BREWER JM (1985) A spectrophotometric method for quantitation of carboxyl group modification of proteins using Woodward's reagent K. Anal Biochem 151: 327-333

SLEBE JC, HERRERA R, HUBERT E, OJEDA A, MACCIONI RO (1983) Fructose 1,6 bis phosphatase: dissociation of AMP cooperativity and AMP inhibition by carbamylation. J Prot Chem 2: 437-443

STARK GR (1972) Modification of proteins with cyanate. Meth Enzymol 25: 579-584

TAKAHASHI K (1968) The reaction of phenylglyoxal with arginine residues in proteins. J Biol Chem 243: 671-6179

TSOU C-L (1962) Relation between modification of functional groups of proteins and their biological activity. I. A graphical method for the determination of the number and type of essential groups. Sci Sinica 11: 1535-1558

WALSH CT (1984) Suicide substrates mechanism-based enzyme inactivators: recent developments. Annu Rev Biochem 53: 493-535

WARWICK PE, D'SOUZA L, FREISHEIM JH (1972) Natural structural variation in enzymes as a tool in the study of mechanism exemplified by a comparison of the catalytic site structure and characteristics of cathepsin B and papain. Biochemistry 11: 3775-3779

WILLENBROCK F, BROCKLEHURST K (1984) A new synthesis of peptides. Biochem J 222: 805-814

WOODWARD RB, OLOFSON RA, MAYER H (1961) A new synthesis of peptides. J Am Chem Soc 83: 1010-1012

YANKEELOV JA Jr (1972) Modification of arginine by diketones. Meth Enzymol 25: 566-579

YONEMITSU O, HAMADA T, KANAOKA Y (1969) Esterification of peptides in aqueous solution. Tetrahedron Lett 1819-1820