Chemical Approaches to the Study of Enzymes

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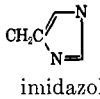
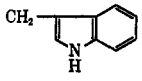
Proteins comprise a remarkably diverse group of compounds. They vary widely, not only in terms of composition and structure, but also in their enormous spectrum of biological functions. Most of these functions are highly specific and constitute a primary means of identifying individual proteins.

Presently, it is almost universally accepted that the function of a protein is directly related to its three-dimensional structure which, in turn, is dictated by its linear sequence of amino acids. Due to molecular folding, amino acid side chains may be brought from distant positions in the linear sequence of the protein into proper spatial alignment and juxtaposition so as to induce the chemical reactivity and surface environment essential for the ultimate manifestation of function.

Protein structure is commonly determined by a combination of chemical sequencing and X-ray crystallographic methods.[1-4] At present such procedures have a number of practical limitations. Some properties of proteins are best examined by chemical procedures. In particular, amino acid residues participating in enzyme function can be identified most effectively by means of chemical modifications. The approach has become increasingly sophisticated in recent years, and efforts are now directed toward discerning not only specific functional residues but also the nature of the environmental factors which render them active. Several recent, general reviews of this subject are available.[5-8]

A large number of reagents can be employed for modification of side-chain residues in proteins,[6,8] and virtually all functional groups are amenable to chemical alteration (Table I). However, most reagents are nonspecific; they react with more than a single residue or even a single class of residues. This may complicate the interpretation of chemical modification studies. Fortunately, certain residues, because of their microenvironment within the three-dimensional structure of the protein, may exhibit reactivities different from those expected on the basis of studies with model compounds. Often, these are the residues which have a direct role in biological function. In fact, the structural factors which generate and condition catalytic function and specificity in enzymes seem to be closely related to those which give rise to such unusual chemical reactivity. It is this characteristic which allows a rather high frequency of unique group labeling in chemical modification studies.

Table I
Reactions for the Modification of Functional Groups in Enzymes

–NHCH(R)CO– functional groups, R	Reactions
$(CH_2)_4NH_2$, ε-amino group of lysine	Acylation, alkylation, arylation, deamination, reaction with reactive carbonyls
$(CH_2)_3NHC(=NH)NH_2$, guanido group of arginine	Condensation with dicarbonyls
$(CH_2)_nCOOH$, n = 1, 2, carboxyl group of aspartic and glutamic acids	Esterification, urea formation with carbodiimides, reduction
CH_2SH, thiol group of cysteine	Oxidation, arylation, alkylation, β elimination, heavy metal derivatives
 imidazole group of histidine	Alkylation, diazonium coupling, iodination, oxidation, photooxidation
$(CH_2)_2SCH_3$, thio ether of methionine	Oxidation, photooxidation, alkylation
$CH_2C_6H_4OH$-*p*, phenolic group of tyrosine	Acylation, alkylation, iodination, nitration, oxidation
 indole group of tryptophan	Alkylation, formylation, oxidation, ozonolysis
CH_2OH, aliphatic hydroxyl group of serine and threonine	Esterification

In instances where 1:1 stoichiometry is not observed, other experimental approaches such as "differential labeling" can be employed. Modification is first performed in the presence of a protective agent (substrate, inhibitor, cofactor, allosteric regulator, etc.), the agent is removed, and the modification is repeated with, *e.g.*, isotopically labeled reagent. Alternatively, if the protein to be modified exhibits a specific binding affinity

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(3) C. H. W. Hirs, *Methods Enzymol.*, **11**, 417 (1967).
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for a particular chemical structure, a chemically reactive side chain can be affixed to that structure in order to bring about covalent bonding to a residue in the vicinity of the binding site. The various maneuvers which can be employed are summarized in Singer's review.[9]

The interpretation of the results of chemical modifications of proteins cannot be based solely on functional parameters. Thus, while loss of enzymatic activity consequent to modification of a particular amino acid side chain is generally assumed to signal the participation of that residue in activity, other possibilities such as denaturation must be considered. For this reason, mild reactions are ordinarily recommended; however, this should not inhibit the courageous from investigating the "unusual" since some proteins can withstand rather extreme conditions.

In this Account, we will not attempt to cover the vast field of chemical modifications of proteins. Rather, we will confine ourselves to a discussion of a few selected means to modify one particular amino acid side chain to best illustrate the principles and considerations involved as well as the type of information which can be obtained.

Modifications of Tyrosyl Residues

Tyrosine is one of the most experimentally accessible residues. Its structure is indicated in Table I. It has spectral characteristics which can be examined in the native protein, providing a means of determining the phenolic dissociation constant. Its physicochemical properties enable it to assume various functions in the mechanism of action of enzymes. It can participate either in substrate binding or in catalysis as a proton donor, as a nucleophile or general base, or as a locus for hydrophobic or charge-transfer interactions. Further, these properties can be influenced markedly by the surrounding environment.

It was not until a few years ago that the susceptibility of tyrosine to chemical modification became fully appreciated; both the aromatic ring and the hydroxyl group can undergo substitution reactions. Some of the more commonly employed reactions are listed in Table I. Because the aromatic ring absorbs light, spectral changes often accompany tyrosyl modification and are valuable in identifying and quantitating the degree of reaction.

The chemical reactions of tyrosyl residues can be divided into four categories.

(**a**) **Ester Formation.** This occurs with simple acid anhydrides,[10,11] acid chlorides,[10,11] acetylimidazole,[12] and phosphoryl and sulfonyl halides.[10,11]

(**b**) **Electrophilic Substitution.** Halogenation,[10,11,13,14] coupling with diazonium compounds,[5,6,15] and nitration with tetranitromethane[16] are typical examples which have been examined in detail and employed widely.

(**c**) **Ether Formation.** Methylation with dimethyl sulfate[10,11] and arylation using fluorodinitrobenzene[17] or cyanuric fluoride[18] has been employed with success in a number of cases.

(**d**) **Oxidation Reactions.** A variety of such reactions are known, including electrolytic oxidation,[19] ultraviolet irradiation,[20] dye-sensitized photooxidation,[21] and enzymatic oxidation with tyrosinase.[22]

Acylation. Acetylation with acetic anhydride or acetylimidazole is a typical example of a suitable tyrosyl chemical modification.[23] The reaction is generally carried out at neutral to slightly alkaline pH (7.5–8.0) with a ratio of reagent to protein of between 10 and 100. Even at 0° the reaction is complete within 1 hr. The pH of the reaction must be controlled, preferentially by an autotitrator (pH-stat), since some buffers influence the course of the reaction.[23] Acetic anhydride reacts with amino, sulfhydryl, phenolic, and hydroxyl groups of proteins. The specificity depends upon the conditions employed and the particular protein. Acetylimidazole is milder and more selective than the anhydride and, even at room temperature, it is less reactive toward amino and aliphatic hydroxyl groups. These circumstances have made it a favored reagent for tyrosyl modification.

O-Acetylation markedly decreases the phenolic absorption at 278 nm ($\Delta E_{278} = 1160$). Exposure to alkali or to neutral hydroxylamine hydrolyzes the ester bond and restores the spectrum; acetylhydroxamic acid can be estimated quantitatively. This provides a means to differentiate, both chemically and biologically, between the acetylation of amino *vs.* phenolic hydroxyl groups.

Cyclic anhydrides, such as succinic anhydride, offer a greater degree of control for limited acylations. The reaction of succinic anhydride with tyrosine reverses spontaneously due to intramolecular catalysis of the hydrolysis of the *O*-succinyl ester bond by the free carboxyl group.[24] However, the rate of deacylation can be increased, for instance, by adding methyl substituents to the methylene carbon atoms or decreased by increasing the number of methylene groups as in glutaric anhydride.[24] Blocking of phenolic hydroxyl groups with chloro- or bromoacetyl groups also is a reversible process due to spontaneous ester hydrolysis.[25]

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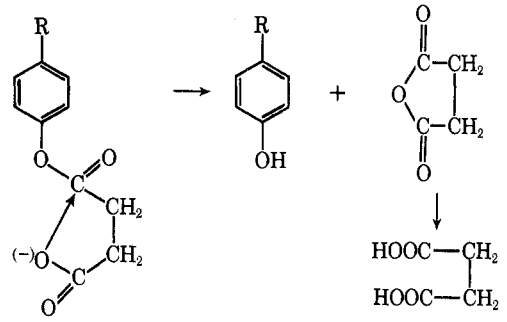

Succinylation of the hydroxyl groups of serine and threonine and of amino groups has also been observed.[26,27] However, these derivatives are relatively stable, and deacylation can only be effected with alkaline hydroxylamine. Furthermore, whereas the products of acetylation of amino and phenolic hydroxyl groups are uncharged, dicarboxylic acid anhydrides introduce an additional negative charge *via* the carboxylate ion. Therefore, these reactions may have different effects on activity, conformation, or degree of aggregation.[28,29]

Nitration. Nitration with tetranitromethane (TNM) has proven to be exceptionally valuable for studying tyrosyl residues in proteins.[30-37] The reagent can be employed under mild conditions—room temperature and pH 8.0. When added in low molecular excess, TNM selectively nitrates ortho to the hydroxyl group of tyrosyl residues. Under these conditions it can also oxidize sulfhydryl groups. The nitration of tyrosine is dependent upon pH, being slow or absent at pH 6 and very rapid at pH 9. Nitration and thiol oxidation can often be distinguished by performing the reaction at pH 6 where only oxidation takes place.

Bruice, *et al.*,[38] have investigated the nitration of phenol model compounds by TNM in detail and have suggested the mechanism outlined in Scheme I. TNM forms a charge-transfer complex with phenoxide ion, in aqueous solution, thus accounting for the pH dependence of the reaction. This is followed by a rate-determining electron transfer to generate the phenoxide and nitrite radicals plus the nitroformate anion. Nitration

Scheme I

$$XC_6H_4O^- + TNM \rightleftharpoons \text{charge-transfer complex}$$

$$\text{charge-transfer complex} \overset{\text{rate determining}}{\rightleftharpoons} [XC_6H_4O\cdot + NO_2\cdot] + C(NO_2)_3^-$$

$$NO_2XC_6H_4O^- \leftarrow [XC_6H_4O\cdot + NO_2\cdot] + C(NO_2)_3^-$$

$$[XC_6H_4O\cdot + NO_2\cdot] \rightarrow XC_6H_4O\cdot + NO_2\cdot$$

$$NO_2\cdot + XC_6H_4O^- \rightleftharpoons XC_6H_4O\cdot + NO_2^-$$

$$nXC_6H_4O\cdot \rightarrow \text{coupling products}$$

results from the coupling of these two radicals, but additional reactions are made possible by the coupling of phenolate radicals with each other. Further, nitrite ions are formed by electron transfer between $NO_2\cdot$ and additional phenolate ions.

The reaction of TNM with sulfhydryl groups is thought to proceed by the mehanism given in Scheme II.[39] An intermediate sulfenyl nitrate is formed which,

Scheme II

$$RSH + TNM \rightarrow RSNO_2 + C(NO_2)_3^- + H^+$$

$$RSNO_2 \xrightarrow{RSH} RSSR + NO_2^- + H^+$$

$$RSNO_2 \xrightarrow{H_2O} RSOH + NO_2^- + H^+$$

$$RSOH \xrightarrow{[O]} RSO_2H$$

in the presence of excess thiol, *i.e.*, low molar excesses of TNM, gives the disulfide and nitrite ion. Alternatively it can hydrolyze to give the sulfenic acid which, upon exposure to air, oxidizes to the sulfinic acid. The relative amounts of disulfide and sulfinic acid depend upon reaction conditions, the ratio of TNM to RSH, and the nature of the R group.[39]

When TNM is employed in high molar excesses at pH 8, or if the reaction is carried out at higher pH values, other residues such as histidine, tryptophan, and methionine will react.[39,40] The products of these reactions have not been identified thus far but, in the case of tryptophan, a number of nitrated derivatives have been demonstrated.[33,40]

According to the above schemes, it should be possible to follow the modification of proteins with TNM by measuring the release of nitroformate anion. This species absorbs at 350 nm with an extinction coefficient of 14,400.[41] However, we have found that with a number of proteins the amount of nitroformate produced is greater than that which can be accounted for by tyrosyl modification, sulfhydryl oxidation, or loss of other residues. This suggests the possibility of a catalytic breakdown of TNM. Thus, the appearance of 350-nm absorption can serve only as a qualitative gauge for the reaction of TNM with proteins.

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The free-radical mechanism for nitration can lead to potentially significant side reactions, protein–protein interaction and polymerization. Such cross-linking of proteins has been reported to occur on nitration in a number of instances.[42-44] The formation of nitrite ion, as a by-product, can also cause unwanted side reactions[45] If modification is terminated by acidification, as has been done on occasion, the resultant nitrous acid can (a) modify previously unreacted tyrosyl residues; (b) deaminate lysyl residues; (c) react with thiol and other side-chain groups, and (d) induce polymerization. Thus, it is critical that excess reagent and by-products be removed from the reaction mixture by gel filtration or dialysis prior to lowering the pH. The formation of nitrate esters[39] can provide another mechanism by which protein polymerization may occur. Such active esters could react with, *e.g.*, amino groups leading to inter- and intramolecular peptide bonds.

Diazonium Coupling. Proteins may also be modified by coupling with aromatic diazonium salts. These reagents, long used in the study of proteins, interact readily with tyrosyl, histidyl, and lysyl residues, producing intensely colored products with the first two. Recently it has been observed that, in some proteins, arginine and tryptophan (at the α position) can also couple with diazonium salts.[46,47] An enormous number of diazonium compounds are available, but little attention has been given to the influence of the aromatic residue on the specificity of the reaction. Rather, attempts have been made to define specificity on the basis of absorption spectra. However, lack of residue or spectral specificity has largely rendered such reagents of limited value for purposes of definitive indentification. Nevertheless, under appropriate conditions azo labels have been shown to be attached specifically to tyrosine residues at the active sites of antibodies.[48] Moreover, it is possible to overcome the broad specificity of these reagents by performing successive chemical modifications, *i.e.*, blocking exposed lysyl residues by acylation, thus preventing their subsequent reaction with the diazo reagent.[49]

Besides their lack of residue specificity, diazonium salts suffer some disadvantages in that more than one derivative may be formed with tyrosyl, histidyl, and lysyl residues. In the case of tyrosine, the 3-azo and the 3,5-bisazo derivatives have been identified. Mono coupling proceeds rapidly, while the second step is slower. With histidine, bis coupling to give the 2,4 derivative is quite fast, and it is uncertain if monoazohistidine is the 2 or the 4 derivative. Lysine is generally thought to form a pentazine, but recent evidence[50] would suggest that the triazine may be the predominant species.

Structural and Functional Consequences of Protein Modification

Each of these modification procedures has been employed to specific advantage with a variety of enzymes. Acylation is the only reversible reaction known for tyrosine. This fact led to the initial demonstration that two tyrosyl residues are essential to the catalytic mechanism of carboxypeptidase A.[51] In addition, the ease by which acylation can be measured suggested a method for the determination of "free" and "buried" residues in proteins.[12] These designations, "free" and "buried," are used merely to indicate the susceptibility of certain residues to chemical modification and, hence, are strictly operational. They are not intended to define the location of residues on the interior or exterior of the three-dimensional protein structure, though this could obviously influence reactivity markedly. The chemical reactivity of side-chain groups of proteins is largely determined by local environmental factors such as the charge or polarity of neighboring residues.

Acetylation has helped to identify "regulatory" as well as "catalytic" sites in enzymes. Acetylation of fructose 1,6-diphosphatase from rabbit liver with acetylimidazole has revealed two classes of functional tyrosyl residues.[52] In the first phase of modification, two or three tyrosyl residues are acetylated with no change in catalytic properties. The acetylation of four additional residues is associated with the loss of allosteric inhibition by adenosine monophosphate. Finally, the acetylation of the last few residues abolishes catalytic activity. The last phase is blocked by the substrate while the inhibitor protects the second group of tyrosyl residues.

Nitration affords multiple opportunities for studying tyrosyl residues in proteins. Some of the results of such studies (see ref 53–72) are summarized in Table II. In

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Table II
Consequences of Tyrosyl Nitration in Proteins

Protein	Modification consequences
Aminopeptidase M	Nitration of one tyrosyl residue per monomer causes inactivation[53]
Antibodies to DNP	Nitration of a tyrosyl residue affects the combining site[54]
Arginine kinase	Nitration of a single tyrosyl residue results in a loss of catalytic properties with accompanying structural changes[55]
Aspartate aminotransferase	One tyrosyl residue is nitrated only during the course of catalysis, resulting in "syncatalytic" inactivation[56]
Bovine neurophysin II	Indicates that the tyrosyl residue enters into a more hydrophobic environment on protein–hormone interaction[57]
Chymotrypsin	Two of the four tyrosines (146 and 171) are nitrated in the enzyme, while in the zymogen residue 94 is nitrated as well[58]
Carbonic anhydrase	Nitration reveals the presence of a single "free" tyrosine, but modification is without effect on activity[59]
Carboxypeptidase A	Esterase activity increases and peptidase activity decreases on nitration of a single environmentally sensitive tyrosine[32]
Carboxypeptidase B	Both the esterase and peptidase activities are abolished simultaneously with the modification of one tyrosyl residue[36]
Cytochrome *c*	Nitration of tyrosine-67 abolishes respiratory function and changes spectral properties[35]
Glucose 6-phosphate dehydrogenase	Catalytic function is lost after nitration or acetylation of 2 out of 34 total tyrosyl residues[36]
Glutamate dehydrogenase	Acetylation or nitration of a single tyrosine per subunit leads to desensitization to allosteric inhibition but without any marked changes in enzymic activity[60]
Glycoprotein	Three tyrosyl residues play an essential role in maintaining the reactivity of the protein toward specific antibodies[61]
Hemerythrin	Nitration indicates that three tyrosyl residues provide ligands to each iron[62]
Lysozyme	Nitration of tyrosine-20 and -23 causes some conformational changes, as evident from the increase of susceptibility to tryptic hydrolysis and the exposure of one disulfide bridge[63]
Myoglobin	Nitration suggests that one or both of tyrosines-146 and -151 are present in a reactive region of metmyoglobin[64]
Ornithine carbamyl transferase	Tyrosine may be present in the active site[65]
Ovine hormone	Nitration produces significant structural changes without altering the biological properties[66]
Pepsin	Nitration of four to five tyrosyl residues significantly alters the enzymic activity accompanying some conformational changes[36,67]
Ribonuclease A	Of the six tyrosine residues, three can be nitrated, with differing reactivity, without altering activity[30,68]
Thrombin	Clotting activity is virtually abolished by nitration with a slight decrease in esterase activity[36]
Transferrin	Acetylation and nitration of tyrosyl residues affect the metal-binding properties of these proteins[69]
Trypsin	Nitration has no effect on catalytic activity toward small substrates but decreases considerably that toward macromolecular substrates[43]
Trypsin inhibitor	Nitration of two tyrosyl residues does not affect the trypsin-inhibitor activity, but gives rise to a conformation-dependent, side-chain Cotton effect in the region of nitro aromatic absorption[70]
Staphylococcal enterotoxin	Five tyrosyl residues are "free" and 16 are "buried"[71]
Staphylococcal nuclease	Nitration of tyrosine-85 abolishes activity toward DNA and RNA. In the presence of inhibitor only tyrosine-115 is nitrated without altering DNA activity while RNA activity is reduced by 50%[33]
Subtilisin Carlsberg	Nitration alters the binding capacity so that hydrolysis of clupein and gelatin is facilitated, while hydrolysis of small substrates is not affected[34]
Sulfatase	Eight tyrosyl residues are nitrated while acetylation causes reversible inactivation[72]

many cases modification is quite specific. Thus, the incorporation of about one nitro group per molecule of carboxypeptidase A with a fourfold molar excess of TNM enhances esterase and greatly reduces peptidase activity.[32] The presence of the inhibitor, β-phenylpropionate, during the nitration reaction prevents the changes in activity. Similarly, a low molar excess of tetranitromethane selectively nitrates the tyrosyl residue at position 85 in the extracellular nuclease of *Staphylococcus aureus*, resulting in loss of catalytic activity toward both DNA and RNA.[33] Reaction of the nuclease with TNM in the presence of Ca^{2+} and deoxythymidine 3′,5′-bisphosphate, pdTp, selectively nitrates tyrosine-115, while tyrosine-85 is protected. This product is fully active toward DNA but only half active toward RNA.

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Nitration of tyrosine-67 in horse heart cytochrome *c* has a number of effects: the closed crevice structure of the molecule is distinctly weakened, activity is abolished, and in neutral solution the oxidized form cannot be reduced with ascorbate, nor does it show a 695-nm band.[35] The loss of the 695-nm band indicates that the sulfur atom of methionine-80 is no longer coordinated to the heme iron. Apparently, tyrosine-67 must contribute significantly to the maintenance of the native conformation of the protein. It is of interest that, although it is most readily nitrated, tyrosine-67 is in the interior of the molecule.[73] This may be due to its proximity to positively charged residues which would promote its ionization and, hence, its reactivity. Furthermore, spectral analysis of nitrocytochrome *c* has led to the suggestion that tyrosine-67 may undergo nitration at either ortho position of the phenolic ring to give two different species.[35]

Nitration of aspartate aminotransferase in the presence of the substrate pair, glutamate and α-ketoglutarate, abolishes activity concomitant with the modification of one tyrosyl residue. In the absence of substrate, there is virtually no inactivation while, in the presence of competitive inhibitors or either substrate alone, only slight inactivation occurs. The tyrosyl residue becomes reactive only subsequent to the formation of the enzyme–substrate complex during the actual catalytic process. This syncatalytic (*i.e.*, synchronous with catalysis) inactivation suggests that the tyrosyl residue plays a role in the process of transamination.[56] These observations have provided convincing evidence for the significance of transient conformational changes to the mechanism of action of enzymes.

TNM has also been employed to examine intermediates in reactions of yeast and muscle aldolases.[74] The high molar absorptivity of the product, nitroformate, provides a convenient means of detecting carbanionic enzyme–substrate complexes and, in fact, TNM has been suggested as a general reagent for the titration of carbanions.[75]

The insertion in a protein of a chromophore which absorbs in the visible region of the spectrum, *i.e.*, nitrophenol, provides a number of convenient experimental approaches.

(1) Since the nitrotyrosyl residue can ionize, it may serve to probe the microenvironment of active center residues by means of perturbation spectra and similar methods. Spectral titrations indicate an apparent pK of 6.3 for the nitrotyrosyl residue of mononitrocarboxypeptidase compared with the pK of 7.0 for *N*-acetyl-3-nitrotyrosine. Hence, the specific nitration of a single tyrosyl residue in carboxypeptidase would appear to be related to an abnormally low pK for that residue, perhaps due to features of its chemical environment which induce ionization. Lightly nitrated ribonuclease[68] appears to contain a very small amount of anomalous nitrotyrosine titrating above pH 10. It has been suggested that this might be due to nitration of a buried residue, tyrosine-92. However, tyrosine-92 is not obviously inaccessible in the ribonuclease structure, but it does exhibit abnormal ionization in the native protein.[76] If these observations are correct, it would seem that this residue might be nitrated by a mechanism which differs from that proposed by Bruice, *et al.*[38]

(2) If the group modified is functional, substrates, substrate analogs, and inhibitors may affect its spectral properties. Addition of β-phenylpropionate to nitrocarboxypeptidase shifts the absorption spectrum.[31] Spectral titrations in the presence of this inhibitor indicate a pK for the nitrotyrosyl residue of 7.0 instead of 6.3, suggesting that the reagent alters the immediate chemical environment of the active center nitrotyrosyl residue. Further, studies on the nitration of tyrosyl copolymers have shown that the rate of nitration is a function of the net charge on the tyrosyl copolymer. Thus, if the tyrosyl residues are surrounded by positively charged lysyl groups, the rate of nitration is much faster and the pK of the resultant nitrotyrosyl group much lower than when they are surrounded by negatively charged aspartate or glutamate residues. This may account for the preferential nitration of tyrosine-67 in cytochrome *c*. The specific nitration of tyrosine-115 in staphylococcal nuclease appears to be analogous to the situation in carboxypeptidase A. Thus, in the absence of nucleotide, the nitrotyrosyl residue has an abnormally low pK of 6.4. Addition of pdTp shifts the pK to 7.2, similar to that seen in model compounds. The pK of nitrotyrosyl residue 85 is also low, 6.6, but is altered only slightly by added nucleotide.

The effect of the environment on the pK of nitrotyrosine also has been examined using different solvents. Increasing ionic strength decreases the pK while decreasing polarity increases the pK of the phenolate group. Based on studies with model compounds it was concluded that the effect of β-phenylpropionate and of substrates such as glycyl-L-tyrosine, glycyl-L-leucine, and others on nitrocarboxypeptidase is the result of an apparent shift in the pK of the nitrotyrosyl residue, brought about by a change in local environment.[31] In the native enzyme, the tyrosyl residue was presumed to be in close proximity to a positively charged group which enhances its rate of nitration, reflected in a lowered pK for the resultant nitrotyrosyl residue. On addition of substrate the tyrosyl residue shifts away from the positively charged residue and into a more hydrophobic environment. These deductions from chemical evidence are consistent with X-ray structural data,[77] which have revealed that tyrosine-248, in the active center of carboxypeptidase A, interacts with the positive charge of arginine-145, and on addition of glycyl-L-tyrosine it moves more than 12 Å away from this arginine. This could account for the spectral changes observed on additions of substrates and inhibitors to the nitro enzyme.

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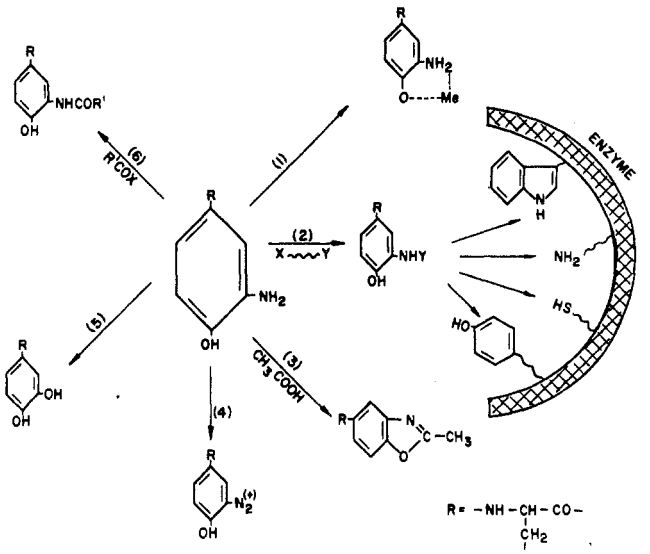
Scheme III



(3) The nitrotyrosyl group, if optically active, might exhibit characteristic extrinsic Cotton effects as additional probes of protein conformation. Reaction of pancreatic trypsin inhibitor with TNM results in both a mono- and a disubstituted derivative nitrated at positions 10 or 10 and 21, respectively, both retaining full trypsin inhibitor activities.[70] Optical rotatory dispersion measurements indicate that nitration does not effect the protein secondary structure but gives rise to a conformation-dependent, side-chain Cotton effect in the region of nitro aromatic absorption. A nitrotyrosine-containing pentadecapeptide isolated from the tryptic digest of the nitrated and oxidized inhibitor does not exhibit such a Cotton effect.[70] Importantly the spectral characteristics represent a guide to labeling and subsequently isolation of nitrotyrosyl peptides.

(4) The nitrotyrosyl residue is a potential site for further modification since it can be reduced to an amino group using sodium hydrosulfite.[78] When a five-to six-fold molar excess of $Na_2S_2O_4$ is added to nitrotyrosyl derivatives at pH's between 6 and 9, nitrotyrosine disappears within a few minutes. The major product is the aminotyrosine derivative. In most proteins, aminotyrosine appears to be generated virtually quantitatively, as determined by amino acid analysis, though in some model compounds, *e.g.*, nitrotyrosine, glycylnitrotyrosine, and *N*-acetyl-3-nitrotyrosine, other by-products have been observed, principally an apparent sulfamic acid derivative.[79] The mechanism of reduction by $Na_2S_2O_4$ is not yet clear, though participation of the SO_2 radical ion in the reduction[80] could explain the formation of these products. Alternatively, or in addition (Scheme III, reaction 3), cyclization followed by hydrolysis might lead to such a product. In fact, yield of the by-product is increased if sodium sulfite is added prior to the reduction of the nitrotyrosine.

Table III
pK' Values for Phenolic Hydroxyl Ionization in Tyrosine and Tyrosyl Derivatives

Compound	pK'
Tyrosine	10.1
3-Aminotyrosine	10.0
3-Acetylaminotyrosine	9.2
3-Succinylaminotyrosine	9.2
3-Azotetrazolyltyrosine	8.8
3,4-Dihydroxyphenylalanine	8.7
3-Iodotyrosine	8.2
3-Nitrotyrosine	7.2
3,5-Diiodotyrosine	6.4

The apparent activation of functional tyrosyl residues by their environment, as evidenced by changes in apparent pK, suggests a possible chemical basis for their catalytic role. Alteration of the phenolic pK can also be brought about by reduction of the nitro to an amino group (Table III).

Reduction does not affect the activities of nitrocarboxypeptidase, but it does affect activity in two other instances. Thus, nitration of *E. coli* alkaline phosphatase reduces hydrolase activity but not phosphotransferase activity. Subsequent reduction of the nitro to the amino enzyme restores hydrolytic activity to that of the native enzyme and increases transferase activity threefold.[81] Reduction of nitronuclease restores some of its activity toward RNA but less of its activity toward DNA.

The introduction of aminophenols into the primary sequence of proteins provides the possibility for further permutations, as shown in Scheme III. Chelation of metal ions (Scheme III, reaction 1) exemplifies the unique properties of aminophenols which may serve a particularly useful function. Several aminophenol metal chelates are colored, for instance, thereby allowing identification of the group, possibly the recognition of vicinal effects, and, perhaps, perturbation through spectral studies. Some metal chelates may prove to be suitable for nmr studies. Such modifications, *i.e.*, introduction of metals, represent an extension of available procedures to prepare derivatives for X-ray diffraction.

Furthermore, the reaction with bifunctional reagents (Scheme III, reaction 2) which leads to cross-linking provides an important tool for chemical determination of the topography within a protein molecule. The selective modification of the amino group can be achieved by performing the first reaction near the pK of this group, *i.e.*, 4.7, conditions under which the phenolic hydroxyl group is not, *e.g.*, acetylated or succinylated, and reaction at the α- and ϵ-amino groups will be very slow if at all.[79] Then, the pH of the reaction mixture, with appropriate dilution to prevent intermolecular cross-linking, can be raised, allowing reaction with other neighboring active side chains (Scheme III, reaction 2).

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This procedure[79] has been used with the nuclease of *Staphylococcus aureus*.[82] Aminotyrosyl nuclease was treated with *p,p'*-difluoro-*m,m'*-dinitrophenyl sulfone or 1,5-difluoro-2,4-dinitrobenzene, which at pH 5 gives exclusive monofunctional substitution at the aromatic amino group. On exposure to pH 9.4 the derivative substituted at aminotyrosine-85 cross-links with tyrosine-115, while the derivative substituted at aminotyrosine-115 cross-links with lysyl residues. The proximity of tyrosine-115 to lysine residues may account for its low pK when nitrated.

o-Aminophenols similar to phenylenediamine tend to form cyclic compounds (Scheme III, reaction 3). This blocks both ionizable groups and allows further investigation of their relationship to function. Conversion to the diazonium salt (Scheme III, reaction 4) offers another possibility for cross-linking. In addition, this modification may prove useful for mapping the active site of an enzyme by affinity labeling, using low and high molecular weight substrates that have been modified at their tyrosyl residue.

Oxidative deamination (Scheme III, reaction 5) is usually catalyzed by metals. The product, 3,4-dihydroxyphenylalanine, can be easily oxidized to the quinone analog either chemically or enzymatically with tyrosinase (*o*-diphenol oxidase).

Finally we wish to indicate how diazonium salts can be useful for studying the relationship between structure and function in enzymes. Low concentrations of 5-diazo-1*H*-tetrazole (DHT) have been found to modify tyrosyl and lysyl residues in carboxypeptidase A.[49] A 200% increase in esterase activity correlates with the modification of a single tyrosyl residue, but peptidase activity is not affected. Higher concentrations of DHT decrease peptidase activity progressively, correlating with the loss of one histidyl residue. Prior acetylation of the tyrosyls results in monoazohistidylcarboxypeptidase lacking peptidase activity but retaining the esterase activity of the native enzyme.

This functional response differs significantly from those observed on nitration of one tyrosyl residue in carboxypeptidase A. The possible roles of various tyrosyl residues in its mechanism were studied further by successive chemical modifications.[32] This approach has led to the suggestion that each of these two tyrosyl residues may play a different role in the mechanism of action of carboxypeptidase A.

As pointed out above, coupling of tyrosyl residues gives colored products which can be used, like the nitrotyrosyl residue, as a probe of the chemical environment of the modified residue.[50,83,84] Modification of about a dozen different proteins with the same diazonium salt results in a series of circular dichroic spectra characteristic of each protein. On the other hand, modification of a single protein with different diazonium salts yields a family of closely related dichroic spectra.[84] This suggests that the structure of the protein plays a major role in determining the spectral details. Hence, circular dichroism can be used to monitor conformational changes accompanying enzymatic processes. For example, the circular dichroic spectrum of arsanilazocarboxypeptidase (formed by coupling with *p*-azobenzenearsonate)[50] contains multiple, extrinsic ellipticity bands with peaks at 530, 430, and 328 nm. On addition of either β-phenylpropionate or glycyl-L-tyrosine, the ellipticity band at 530 nm disappears; changes at shorter wavelengths are also observed. Apparently the binding of substrate and inhibitors alters the conformation of an azotyrosyl residue. Similarly, changes in azotyrosyl ellipticity have been employed to follow the activation of zymogens, such as arsanilazoprocarboxypeptidase[83] and arsanilazochymotrypsinogen.[85]

The coupling of diazonium salts to proteins offers yet another experimental advantage. Azo derivatives of tyrosyl and histidyl residues, like nitrophenol, can be reduced with $Na_2S_2O_4$, yielding the aminotyrosyl and aminohistidyl derivatives, respectively. The opportunities for further reactions with aminotyrosine have been discussed already (Scheme III), and analogous possibilities exist for aminohistidine. Since monoazotyrosine is destroyed by acid hydrolysis, it cannot be detected by amino acid analysis. However, direct quantitation can be achieved *via* reduction and determination of aminotyrosine.[78]

Concluding Remarks

We have attempted to present in this discussion some representative examples of chemical approaches to the study of enzymes. While we have concentrated on tyrosine, it should be clear that similar considerations apply to the modification of other functional residues. There is by now a wide selection of reagents available for use under mild conditions; many more are likely to be discovered and can be utilized profitably. Further, we have confined this discussion to reactions which occur in aqueous solutions and have not touched upon the special advantages which may accrue from the use of nonaqueous solvents and modification of crystalline enzymes or enzymes bound to membranes. The entire field of enzyme synthesis, with the potential for inserting altered amino acids into defined locations, has emerged only recently, but offers many exciting additional possibilities. Nevertheless, we hope that the examples selected will provide insight and stimulation to generate investigative approaches which may ultimately lead to a chemical definition of the mechanisms of enzyme action.

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