Techniques in PROTEIN MODIFICATION

Roger L. Lundblad

Techniques in PROTEIN MODIFICATION

Roger L. Lundblad

CRC Press
Boca Raton Ann Arbor London Tokyo

Library of Congress Cataloging-in-Publication Data

Lundblad, Roger L.
Techniques in protein modification / author, Roger L. Lundblad.
p. cm.
Includes bibliographical references and index.
ISBN 0-8493-2606-0
1. Proteins—Chemical modification. I. Title.
QP551.L883 1994
574.19'245—dc20
DNLM/DLC
for Library of Congress 94-11646
CIP

International Standard Book Number 0-8493-2606-0
Library of Congress Card Number 94-11646
Printed in the United States of America 2 3 4 5 6 7 8 9 0
Printed on acid-free paper

PREFACE

This book is an extension of the previous editions of *Chemical Reagents for Protein Modification* (CRC Press, Boca Raton, FL). Since it has been the author's experience that the previous editions were extensively used in the laboratory, this current version has been designed for the laboratory worker. Thus, a majority of the illustrations from the previous volumes have been deleted, as the author did not consider them essential to the laboratory research phase. Material regarding sources of materials and information have been included.

While a consideration of major biochemical journals would suggest that the use of chemical modification to study the relationship between structure and function in proteins is no longer an active area, the reader is reminded that the chemical characterization of protein is still a major concern for biopharmaceutical companies, and there is an active area in the development of site-specifically modified proteins as therapeutics.

A major portion of this book has been written in various airports and at various altitudes. The support of numerous colleagues is greatly appreciated. In particular, the author is indebted to Professor Charles Craik of the University of California at San Francisco and Professor Bryce Plapp of the University of Iowa, Iowa City for many useful discussions regarding protein engineering and functional group reactivity. The author is also indebted to Professor Ralph Bradshaw of the University of California at Irvine for many entertaining hours of discussions concerning both protein chemistry and the theoretical aspects of Duke-Carolina basketball. Finally, the fifth floor of Flexner Hall at Rockefeller University, New York continues to be a dominant force.

THE AUTHOR

Roger L. Lundblad, Ph.D., is Director of Science and Technology Development in the Hyland Division of Baxter Biotech Group of Baxter Healthcare in Duarte, California. He is also Adjunct Professor of Pathology in the School of Medicine and Adjunct Professor of Periodontics in the School of Dentistry at the University of North Carolina at Chapel Hill.

Dr. Lundblad received his undergraduate education at Pacific Lutheran University in Tacoma, Washington and his Ph.D. degree in biochemistry at the University of Washington in 1965. Before joining Baxter Biotech in 1988, Dr. Lundblad was a full-time faculty member of the University of North Carolina at Chapel Hill. Prior to the University of North Carolina, Dr. Lundblad was a Research Associate in the Moore-Stein group at the Rockefeller University in New York City. Dr. Lundblad has received awards from Pacific Lutheran University, the University of North Carolina, and Baxter Biotech in recognition of his research accomplishments.

Dr. Lundblad's research interests are in the manufacture and characterization of protein biotherapeutics, the role of proteases in biological regulation and wound healing, and the solution chemistry of proteins.

TABLE OF CONTENTS

Chapter 1

SITE-SPECIFIC CHEMICAL MODIFICATION OF PROTEINS

This book is intended to describe laboratory techniques in the site-specific chemical modification of proteins. While such techniques are not used in current protein research as extensively as 20 years ago, such technology is still useful in the characterization of proteins.[1–20] In addition, site-specific chemical modification has been used to produce biotherapeutics.[21,22] The current presentation is not intended to be encyclopedic, and the reader is referred to other more comprehensive works.[23–27] In addition, there are several volumes of *Methods in Enzymology*[28–30] which are extremely useful. The reader is also referred to Appendix I for information concerning the availability of various reagents, addresses of suppliers, and a list of current journals active in publishing the results of site-specific chemical modification studies.

The specific purpose of this chapter is to briefly introduce the concept of site-specific chemical modification including methods of characterizing the product of chemical modification reactions. *Site-specific chemical modification is strictly defined as a process which yields a stoichiometrically altered protein with the quantitative covalent derivatization of a single, unique amino acid residue without either modification of any other amino acid residue or conformational change.* In fact, this objective is rarely obtained because there are several major problems which confound this goal. First, few reagents are specific for the modification of a single functional group (Table 1). Second, even with a reagent which appears to be functional group specific, modification of only one of several residues within a functional group class is unlikely except where the specific residue is uniquely reactive. An example of this is provided by the reactivity of histidine residues at the active sites of enzymes.[31–45] There are also examples where only a small fraction of a given amino acid residue will react. An example of this is provided by the modification of lysyl residues by pyridoxal-5′-phosphate.[46–50] Finally, it is unlikely that the site-specific modification of a protein can be achieved without any conformational change.[51–57]

Establishing the stoichiometry of modification is a relatively straightforward process. First, the molar quantity of modified residue is established by analysis. This could be spectrophotometric as, for example, with the trinitrophenylation of primary amino groups, the nitration of tyrosine with tetranitromethane, or the alkylation of tryptophan with 2-hydroxy-5-nitrobenzyl bromide or by amino acid analysis to determine either the loss of a residue as, for example, in photooxidation of histidine and the oxidation of the indole ring of tryptophan with *N*-bromosuccinimide or the appearance of a modified residue such as with *S*-carboxymethylcysteine or N^1- or N^3-carboxymethylhistidine. In the situation where spectral change or radiolabel incorporation is used to

Table 1
DISSOCIATION CONSTANTS FOR NUCLEOPHILES IN PROTEINS

Potential nucleophile	pKa
Carboxyl	4.6
Imidazole	7.0
Sulfhydryl	7.0
α-Amino	7.8
Phenolic hydroxyl	9.6
ε-Amino	10.2

establish stoichiometry, analysis must be performed to determine that there is not a reaction with another amino acid. For example, the extent of oxidation of tryptophan by *N*-bromosuccinimide can be determined spectrophotometrically, but amino acid analysis is *required* to determine if modification has also occurred with another amino acid such as histidine.

In case of the site-specific chemical modification of a protein, it must be established that the modification of one residue mole per mole of protein (or functional subunit) has occurred without modification of another amino acid (e.g., modification has only occurred with lysine and not with tyrosine). The reaction pattern of a given reagent with free amino acids or amino acid derivatives does not necessarily provide the basis for reaction with such amino acid residues in protein. Furthermore, the reaction pattern of a given reagent with one protein cannot necessarily be extrapolated to all proteins. The results of a chemical modification can be markedly affected by reaction conditions (e.g., pH, temperature, solvent and/or buffer used, degree of illumination, etc.). Establishment of stoichiometry does not necessarily mean that this modification has occurred at a unique residue (unique in terms of position in the linear peptide chain — not necessarily unique with respect to reactivity). It is, of course, useful if there is a change in biological activity (catalysis, substrate binding, ion binding, etc.) which occurs concomitant with the chemical modification. Ideally, one would like to establish a direct relationship (i.e., 0.5 mol/mol of protein with 50% activity modification; 1.0 mol/mol of protein with 100% activity modification). More frequently, one might have the situation where there are several moles of a given amino acid modified.[58] In some of these situations it is possible to fractionate the protein into uniquely modified species. The separation of carboxy-methyl-HIS^{12}-pancreatic ribonuclease from carboxy-methyl-HIS^{119}-pancreatic ribonuclease is a classic example of this type of a situation.[58] More recently, it has been possible to separate various derivatives of lysozyme obtained from the modification of carboxyl groups.[59] Frequently, however, while there is good evidence that multiple modified

species are obtained as a result of the reaction, it is not possible to separate apparently uniquely modified species. In the reaction of tetranitromethane with thrombin,[60] apparent stoichiometry of inactivation was obtained with equivalent modification of two separate tyrosine residues (Tyr 71 and Tyr 85 in the B chain) and it was not possible to separate these derivatives.

Indirect support of a site-specific modification can be obtained from a consideration of the functional consequences. In a situation where there are clearly multiple sites of reaction which can be distinguished by analytical techniques, the approach advanced by Ray and Koshland can still be useful.[61] This analysis is based on establishing a relationship between the rate of loss of biological activity and the rate of modification of a single residue. With current technology, it would probably be faster and easier to systematically examine the effect of alanine scanning.[62,63]

The statistical approach advanced by Tsou[64–66] is based on establishing a relationship between the number of residues modified and the change in biological activity. This approach is quite valuable when it is difficult to accurately determine the rate of functional group modification as for example with *N*-bromosuccinimide or 2-hydroxy-5-nitrobenzyl bromide. In Tsou's least complicated example, the biologically essential groups are all of the same type and both essential and nonessential groups are modified at the same rate. Assuming that the modification of any essential group results in the loss of activity, the fraction of biologically active protein remaining will be equal to the fraction of activity remaining (denoted as a). In the situation where there is a single essential group, the fraction of essential groups remaining after any period of modification (denoted as x_c) will be equal to a. In the situation where the number of essential groups is i (by definition greater than 1) among all functional groups of type X, the fraction of each essential group remaining after a period of modification will be x_c. Only those proteins which have all their essential groups intact will retain full activity. Therefore,

$$a = x_c^i \quad \text{or} \quad a^{1/i} = x$$

When all groups of type X react at the same rate, then x_c will be equal to the fraction of the overall fraction of unmodified type X groups and

$$a^{1/i} = x$$

The use of this approach requires the plotting of log a vs. log x; the slope of the resulting line yields i. A number of investigators plot a (activity) vs. m (residues modified).

Horiike and McCormick[67] have explored the approach of relating changes in activity to extent of chemical modification. These investigators state that the original concepts which form the basis of this approach are sound, but that extrapolation from a plot of activity remaining vs. residues modified is not

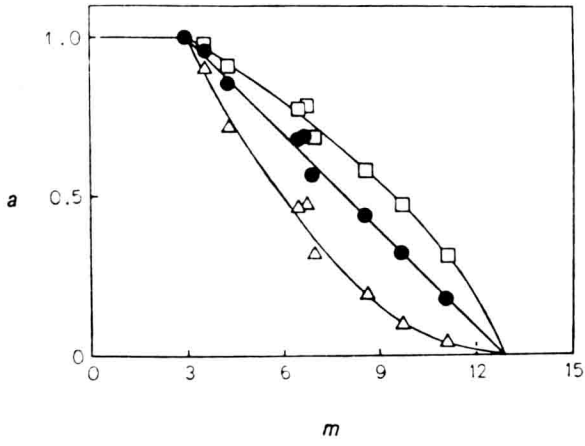

FIGURE 1. Tsou plot for the modification of pepsin with trimethyloxonium fluoroborate. Shown is a plot of (a) vs. (m) where a is the remaining catalytic activity and m is the number of methyl groups incorporated from reaction with trimethyloxonium fluoroborate. The line for i = 2 (●) is the least-squares straight line for these points. The lines for i = 1 (△) and i = 3 (□) are the theoretical curves based upon the i = 2 line. (From Paterson, A. K. and Knowles, J. R., *Eur. J. Biochem.*, 31, 510, 1972. With permission.)

necessarily sound. Such extrapolation is only valid if the "nonessential" residues react much slower (rate at least 100 times slower). Given a situation where all residues within a given group are equally reactive toward the reagent in question, the number of essential residues obtained from such a plot is correct only when the total number of residues is equal to the number of essential residues which is, in turn, equal to 1.0. However, it is important to emphasize that this approach is useful when there is a difference in the rate of reaction of an *essential* residue or residues and all other residues in that class as is the example in the modification of histidyl residues with diethyl pyrocarbonate in lactate dehydrogenase,[68,69] and pyridoxamine-5′-phosphate oxidase.[70] Some examples of the application of Tsou plots to specific chemical modification are presented in Figures 1 to 3.

A major advantage in relating changes in "activity" to a specific chemical modification is being able to demonstrate that the reversal of modification (see Figures 4 and 5) is directly associated with the reversal of the change(s) in biological activity. Demonstrating that the "effects" of a specific chemical modification are reversible lends support *against* the argument that such "effects" are a result of irreversible and "nonspecific" conformational change.

It is useful to consider some factors which influence the reactivity of nucleophilic centers in proteins. From a consideration of the three-dimensional structure of proteins the majority of polar amino acids (i.e., Lys, Arg, Gly, Asp) are located on the exterior surface of the molecule, while the majority of the hydrophobic (nonpolar) residues are located in the interior of these molecules.

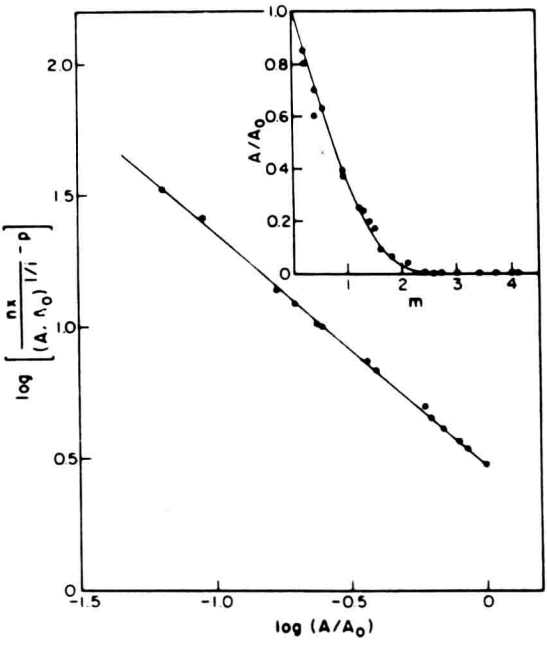

FIGURE 2. Tsou plot for the modification of pyridoxamine phosphate oxidase by diethylpyrocarbonate. The data were plotted using the following equation:

$$\log\left[\frac{nx}{A/A_o^{1/i}} - p\right] = \log(n - p) + \left(\frac{\alpha - 1}{i}\right)\log(A/A_o)$$

where A/A_o is the fraction of enzyme molecules retaining full activity, n is the number of modifiable residues of type X consisting of p residues, of which i are essential, react with the reagent at a pseudo-first-order rate constant k_1 and n – p residues which are not essential reacting at a pseudo-first-order rate constant $k_2(-\alpha k_1)$, and x is the number of residues remaining after reaction with reagent. The data are plotted assuming that in the above equation n = 4 and p = i = 1. The inset describes the relationship between the number of histidyl residues modified per mole of enzyme (m) and A/A_o. (From Horiike, K., Tsuge, H., and McCormick, D. B., *J. Biol. Chem.*, 254, 6638, 1979. With permission.)

Thus, a gradient of polarity (dielectric constant) will exist going from the surface of the protein into the interior. Such a gradient could also be considered to exist in "pocket-like" indentations on the protein surface. For example, the area immediately adjacent to the active site and substrate site S_1 in thrombin is definitely hydrophobic with respect to the surrounding environment and the

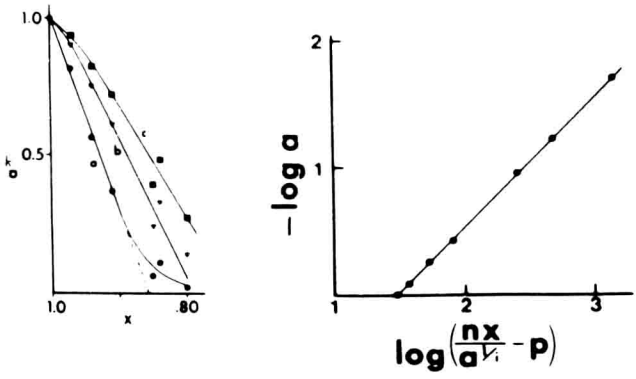

FIGURE 3. The left figure is a Tsou plot for the modification of arginine residues in transketolase by phenylglyoxal. The abscissa is a ratio of unmodified arginine residues to the total number of arginine residues. The ordinate is where a is the fraction of activity remaining and i is a small integer. A linear segment is generated in the case where p residues including i essential residues react at a rate k and n – p residues react with rate αk. The following equation adapted from Tsou:

$$nx = pa^{1/i} + (n - p)\, a^{\alpha/i}$$

reduces to

$$a^{1/i} = \frac{nx - (n - p)}{p}$$

when $\alpha \ll 1$. This gives a straight line with the x intercept equal to n – p/n. This is represented by the extrapolation of the linear portion of the curve to the x axis: a, i = 1, b, i = 2, c, i = 3. The best fit is provided by i – 1 and in this case p = 4 to 5 residues/active site. The right figure describes the determination of α (the constant relating the reaction rates of rapidly and slowly reacting residues). The following equation

$$nx = pa^{1/i} + (n - p)\, a^{\alpha/i}$$

adapted from Tsou can be rearranged as

$$\log (nx/a^{1/i} - p) = \log (n - p) + [(\alpha - 1)/i] \log a,$$

where i and p are determined as described above. α is determined from the slope of the resulting line. In this situation, α = 0.023 implying that the rapidly reacting residues have a rate constant approximately 40-fold greater than the slowly reacting residues. (From Kremer, A. B., Egan, R. M., and Sable, H. Z., *J. Biol. Chem.*, 255, 2405, 1980. With permission.)

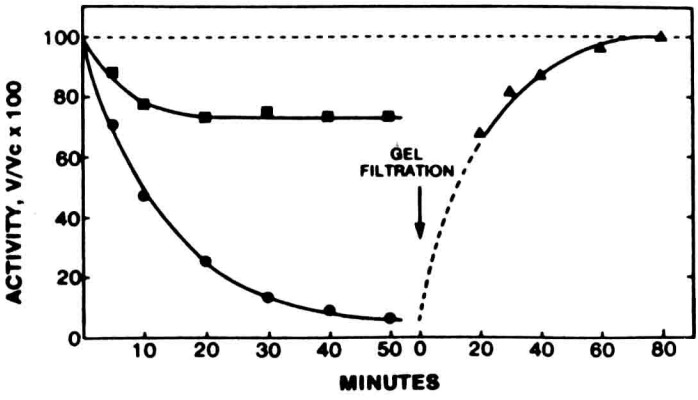

FIGURE 4. The reversible modification of pyridoxamine-5′-phosphate oxidase by 2,3-butanedione. The enzyme (2.1 μ*M*) was incubated with 10 m*M* 2,3-butanedione in the presence of 5 μ*M* flavin mononucleotide (FMN) in either 50 m*M* potassium borate, pH 8.0 (●) or 50 m*M* potassium phosphate, pH 8.0 (■). The reaction mixture in borate was passed over a G-25 Sephadex column and assayed for enzyme activity (▲). The arrow indicates the time at which the reaction mixture in borate was applied to the gel filtration column. (From Choi, J.-D. and McCormick, D. B., *Biochemistry*, 20, 5722, 1981. With permission.)

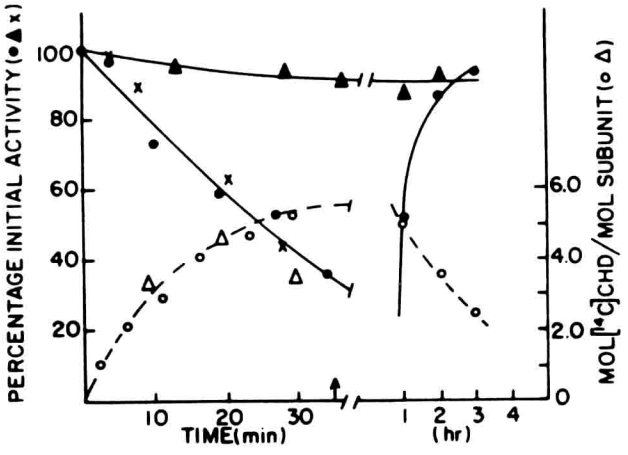

FIGURE 5. The reversible modification of ADP-glucose synthetase by 1,2-cyclohexanedione (CHD). The enzyme was incubated with 10 m*M* [^{14}C] CHD in the presence (•, ○) or absence (x, △) of 50 m*M* sodium tetraborate. The control (▲) was incubated in the absence of CHD. Portions were removed at the indicated times for the determination of incorporated radioactivity (open symbols) or fructose diphosphate-stimulated enzyme activity (closed symbols). The arrow indicated the time of addition of 0.2 *M* hydroxylamine. (From Carlson, C. A. and Preiss, J., *Biochemistry*, 21, 1929, 1982. With permission.)

aqueous solution. This is best demonstrated by the increase in the fluorescence of *N*-α-dansyl-L-arginine-*N*-(3-ethyl-1,5-pentanediyl) amide upon binding to the active-site region.[71]

It should follow from the above discussion that the surface of a globular protein is definitely not homogeneous with respect to electrical charge or, more critically for our consideration, with respect to dielectric constant. As a result of this lack of homogeneity, a variety of surface polarities will surround the various functional groups. The physical and chemical properties of any given functional group will be strongly influenced by the nature (e.g., polarity) of the local microenvironment. Changes in the polarity of the microenvironment can have a profound effect on the dissociation of acids. For example, consider the effect of the addition of an organic solvent, ethyl alcohol, on the pKa of acetic acid. In 100% H_2O, acetic acid has a pKa of 4.70. The addition of 80% ethyl alcohol results in an increase of the pKa to 6.9. In 100% ethyl alcohol the pKa of acetic acid is 10.3. This is particularly important in considering the reactivity of nucleophilic groups such as amino groups, cysteine, carboxyl groups, and the phenolic hydroxyl group. In the case of the primary amines present in protein, these functional groups are not reactive except in the free base form. In other words, the proton present at neutral pH must be removed from the ε-amino group of lysine before this functional group can function as an effective nucleophile. A listing of the "average" pKa values for the various functional groups present in protein is also given in Table 1.

Other factors which can influence the pKa of a functional group in a protein include hydrogen binding with an adjacent functional group, the direct electrostatic effect of the presence of a charged group in the immediate vicinity of a potential nucleophile, and direct steric effects on the availability of a given functional group.

There is another consideration which can in a sense be considered either a cause or consequence of microenvironmental polarity. This has to do with the functional groups/environment immediately around the nucleophilic species in question. These are the "factors" that can cause a "selective" increase (or decrease) in reagent concentration in the vicinity of a potentially reactive species. The most clearly understood example of this is the process of affinity labeling. Another situation can be related to the differences in polarity of the microenvironment around a nucleophilic center. There is also the consideration that a charged reagent can be either attracted to or repelled from the vicinity of a nucleophilic center. This is easily demonstrated by the differences in the comparative rates of modification of the active-site cysteinyl residue by chloroacetic acid and chloroacetamide in papain.[71a]

Another major use of chemical modification has been in the determination of the primary structure of proteins. This includes reagents such as cyanogen bromide for the chemical cleavage of specific peptide bonds, citraconic anhydride for the reversible blocking of lysine residues to restrict tryptic cleavage to arginine residues, and the reversible blocking of arginine residues with 1,2-cyclohexanedione to restrict tryptic cleavage to lysine residues.

The use of specific chemical modification to study changes in the environment has been studied over the past 30 years. The study of Kirtley and Koshland[72] provided the basis for the concept of using "reporter" groups to study changes in the microenvironment surrounding a site of modification. This study used 2-bromoacetamido-4-nitrophenol to modify a limited number of sulfhydryl groups in glyceraldehyde-3-phosphate dehydrogenase. The modified protein has a λ_{max} at 390 nm ($\varepsilon = 7100\ M^{-1}\ cm^{-1}$) between pH 7.0 and 7.6. The addition of the coenzyme NAD caused a marked change in the spectral properties (decrease in absorbance at approximately 375 nm and increase in absorbance at approximately 420 nm) of the modified enzyme which is consistent with a change in the microenvironment around the modified residue (increase in polarity of medium which results in increased formation of the nitrophenolate ion). The reaction of 2-hydroxy-5-nitrobenzyl bromide with tryptophanyl residues to yield the 2-hydroxy-5-nitrobenzyl derivative[73] and the reaction of tetranitromethane with tyrosyl residues[74] to form the 3-nitrotyrosyl derivative was extensively used to study microenvironmental changes in the modified proteins. Spin-labeled reagents have also been useful.[75] One early study used spin-labeled derivatives of diisopropylphosphorofluoridate to study the active site environment of trypsin.[76] Subsequent studies used various spin-label derivatives (piperidinyl nitroxide, pyrrolidinyl nitroxide and pyrrolinyl nitroxide substituent groups) of phenylmethylsulfonyl fluoride to compare microenvironments surrounding the active sites in α-chymotrypsin and trypsin.[77,78] These reagents have been more recently used to study the active site of thrombin.[79,80] The preparation of spin-labeled pepsinogen has been reported.[81] This study used a *N*-hydroxy succinimide ester derivative, 3-[[(2,5-dioxo-1-pyrrolidiny)oxyl] carbonyl]-2,5,-dihydro-2,2,5,5-tetramethyl-1H-pyrrolyl-1-oxy, to modify lysyl residues in pepsinogen. Coupling was accomplished at pH 7.0 (0.1 *M* sodium phosphate) for 7 h at 22°C resulting in the derivatization of approximately three amino groups.

REFERENCES

1. **Nagel-Starczynowska, G. and Kaletha, K.,** AMP-Deaminase from human uterine smooth muscle: the effect of DTNB treatment on kinetic and regulatory properties of the enzyme, *Biochim. Biophys. Acta*, 1164, 261, 1993.
2. **Liao, T.-H., Ho, H.-C., and Abe, A.,** Chemical modification of bovine pancreatic deoxyribonuclease with phenylglyoxal — the involvement of Arg-9 and Arg-41 in substrate binding, *Biochim. Biophys. Acta*, 1079, 335, 1993.
3. **Esmann, M., Sar, P. C., Hideg, K., and Marsh, D.,** Maleimide, iodoacetamide, indanedione, and chloromercuric spin label reagents with derivatized nitroxide rings as ESR reporter groups for protein conformation and dynamics, *Anal. Biochem.*, 213, 336, 1993.
4. **Turk, T. and Macek, P.,** The role of lysine, histidine and carboxyl residues in biological activity of equinatoxin II, a pore forming polypeptide from the sea anemone Actinia equina L, *Biochim. Biophys. Acta*, 1119, 5, 1992.

5. **Turk, T., Macek, P., and Gubensek, F.,** The role of tryptophan in structural and functional properties of equinatoxin II, *Biochim. Biophys. Acta,* 1119, 1, 1992.
6. **Nureki, O., Kohno, T., Sakamoto, K., Miyazawa, T., and Yokoyama, S.,** Chemical modification and mutagenesis studies on zinc binding of aminoacyl-tRNA syntheses, *J. Biol. Chem.,* 268, 15368, 1993.
7. **Xia, C., Meyer, D. J., Chen, H., Reinemer, P., Huber, R., and Ketterer, B.,** Chemical modification of GSH transferase P1-1 confirms the presence of Arg-13, Lys-44 and one carboxylate group in the GSH-binding domain of the active site, *Biochem. J.,* 293, 357, 1993.
8. **Clark, S. and Konstantopoulos, N.,** Sulfydryl agents modulate insulin- and epidermal growth factor (EGF)-receptor kinase via reaction with intracellular receptor domains: differential effects on basal versus activated receptors, *Biochem. J.,* 292, 217, 1993.
9. **Hsieh, P.-C., Shenoy, B. C., Haase, F. C., Jentoft, J. E., and Phillips, N. F. B.,** Involvement of tryptophan(s) at the active site of polyphosphate/ATP glucokinase from Mycobacterium tuberculosis, *Biochemistry,* 32, 6243, 1993.
10. **Sellak, H., Franzini, E., Hakim, J., and Pasquier, C.,** Mechanism of lysozyme inactivation and degradation by iron, *Arch. Biochem. Biophys.,* 299, 172, 1992.
11. **Karlstom, A. R., Shames, B. D., and Levine, R. L.,** Reactivity of cysteine residues in the protease from human immunodeficiency virus: identification of a surface-exposed region which affects enzyme function, *Arch. Biochem. Biophys.,* 304, 163, 1993.
12. **Shen, S. and Strobel, H. W.,** Role of lysine and arginine residues of cytochrome P450 in the interaction between cytochrome P4502B1 and NADPH-cytochrome P450 reductase, *Arch. Biochem. Biophys.,* 304, 257, 1993.
13. **Skawinski, W. J., Adebodum, F., Cheng, J. T., Jordan, F., and Mendelsohn, R.,** Labeling of tyrosines in proteins with [15N]tetranitromethane, a new NMR reporter for nitrotyrosines, *Biochim. Biophys. Acta,* 1162, 297, 1993.
14. **Cutruzzola, F., Ascenzi, P., Barra, D., Bolognesi, M., Menegatti, E., Sarti, P., Schnebli, H.-P., Tomova, S., and Amiconi, G.,** Selective oxidation of Met-192 in bovine alpha-chymotrypsin. Effect on catalytic and inhibitor binding properties, *Biochim. Biophys. Acta,* 1161, 201, 1993.
15. **Siksnys, V. and Pleckaityte, M.,** Role of the reactive cysteine residue in restriction endonuclease Cfr9I, *Biochim. Biophys. Acta,* 1160, 199, 1992.
16. **Thompson, S. A. and Fiddes, J. C.,** Chemical characterization of the cysteines of basic fibroblast growth factor, *Ann. N. Y. Acad. Sci.,* 638, 78, 1991.
17. **Reyes, A. M., Bravo, N., Ludwig, H., Iriarte, A., and Slebe, J. C.,** Modification of Cys-128 of pig kidney fructose 1,6-disphosphatase with different thiol reagents: size dependent effect on the substrate and fructose-2,6-bisphosphate interaction, *J. Prot. Chem.,* 2, 159, 1993.
18. **Chang, G.-G., Satterlee, J., and Hsu, R. Y.,** Essential sulfydryl group of malic enzyme from Escherichia coli, *J. Prot. Chem.,* 12, 7, 1993.
19. **Kobayashi, R., Kanatani, A., Yoshimoto, T., and Tsuru, D.,** Chemical modification of neutral protease from Bacillus subtilis var. amylosacchariticus with tetranitromethane: assignment of tyrosyl residues nitrated, *J. Biochem.,* 106, 1110, 1993.
20. **Alon, R., Bayer, E. A., and Wilchek, M.,** Affinity cleavage of cell surface antibodies using the avidin-biotin system, *J. Immunol. Meth.,* 165, 127, 1993.
21. **Smith, R. A. G., Dewdney, J. M., Fears, R., and Poste, G.,** Chemical derivatization of therapeutic proteins, *Trends Biotechnol.,* 11, 297, 1993.
22. **Poznansky, M. J.,** Soluble enzyme-albumin conjugates: new possibilities for enzyme replacement therapy, *Meth. Enzymol.,* 137, 566, 1988.
23. **Lundblad, R. L. and Noyes, C. M.,** *Chemical Reagents for Protein Modification,* CRC Press, Boca Raton, FL, 1984.
24. **Lundblad, R. L.,** *Chemical Reagents for Protein Modification,* 2nd ed., CRC Press, Boca Raton, FL, 1991.