MASTER

- I. The Use of m- and p-Azidobenzamidines, 4-Fluoro-3-nitro-phenylazide, and 3-Azido-1,2,4-triazole as Photoaffinity Probes of Tryptic Binding Site Conformation.
- II. Analysis of Tryptophan in Proteins by an Acidic Reaction of 3-Diazonium-1,2,4-Triazole

by

Michael C. DeTraglia

Submitted in Partial Fulfillment
of the
Requirements for the Degree

DOCTOR OF PHILOSOPHY

Supervised by: John S. Brand

Department of Radiation Biology and Biophysics
University of Rochester Medical Center
Rochester, New York

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1979

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VITAE

Michael Charles DeTraglia was born on [REDACTED], in Utica, New York. He attended elementary and secondary schools in Utica, graduating from Thomas R. Proctor High School in June, 1970. He entered Utica College of Syracuse University in September, 1970, studying under the tenure of a New York State Regents Scholarship. During his undergraduate training, he was twice recipient of summer research fellowships from the Masonic Foundation, and studied diseased-state effects on human erythrocyte fragility under the supervision of Dr. Lawrence C. Cerny at the Masonic Medical Research Laboratory, Utica. He was elected to Phi Kappa Phi and graduated Summa cum Laude, receiving a Bachelor of Science degree in Chemistry in May, 1973. In September, 1973, he began graduate studies in the Chemistry Department at the University of Wisconsin, Milwaukee. There he studied under the support of a Wisconsin Alumni Research Foundation fellowship and served in both research and teaching assistant positions. His Master's dissertation "Coenzyme Binding and Subunit Interactions in Liver Alcohol and Lactate Dehydrogenases" was supervised by Dr. James T. McFarland. He received a Master of Science degree in Chemistry in May, 1976. In September, 1975, he entered graduate training at the University of Rochester, where he studied under the supervision of Drs. Andrew M. Tometsko in the Department of Biochemistry and John S. Brand in the Department of Radiation Biology and Biophysics, receiving departmental fellowship support. He was awarded a Master of Science degree in Biophysics in December, 1977, and continued to study in the Department of Radiation Biology and Biophysics until the completion of the requirements for the Doctor of Philosophy degree.

The author was married on October 4, 1975, to the former Natalie Ellen Purdy of Canandaigua, New York.

to West Virginia...

in defiance

ABSTRACT

Meta- and para-azidobenzamidine have been prepared and evaluated as photoaffinity labels. The compounds inhibit trypsin reversibly in the dark and are competitive with substrate binding. Upon photolysis, irreversible noncompetitive inhibition is observed and is dependent upon concentration, photolysis time, and pH. Specificity of the probes is indicated by experiments in which p-tosyl-L-arginine methyl ester, a trypsin substrate, is used to protect against photoinactivation.

Evaluation of the pH dependence of photoaffinity labeling reveals that the probes are capable of producing varying degrees of irreversible inactivation of trypsin depending upon the pH maintained during photolysis. This phenomenon is caused by the ability of the reagents to translate the affinity of complement binding sites at any pH to a proportionate level of photoinactivation upon photolysis. That the degree of photoinactivation observed with these inhibitors represents a measure of specific binding affinity versus pH is corroborated by 1) the enzyme's activity profile, 2) ORD spectra, and 3) affinity versus pH data obtained for m-ABA by UV spectral titration. In contrast, photolytic inhibition by 4-fluoro-3-nitrophenylazide shows maxima at pH 3 and 9 with little inhibition at pH 6-7. This demonstrates 1) that hydrophobic sites are revealed at pH extremes (enzyme denaturation) and 2) that pH extremes alone are not deterrent to covalent nitrene attachment. These results demonstrate that under controlled conditions photoaffinity techniques may be used to probe changes in target site affinity as a function of pH.

Unlike conventional aryl diazonium reagents which are reactive at alkaline pH, 3-diazonium-1,2,4-triazole (3-DT) has been found to react with a variety of proteins at acid pH. The rate of 3-DT coupling to the indole nucleus has been shown to increase exponentially from $< 200 \text{ M}^{-1} \text{ min.}^{-1}$ at pH 6 to $> 2400 \text{ M}^{-1} \text{ min.}^{-1}$ at pH 2.5. In experiments using trypsin, 3-DT was found to cause loss in enzymatic activity at acidic as well as alkaline pH. Furthermore, amino acid analysis has demonstrated that the acidic reaction with trypsin resulted in the modification only of tryptophan. A scintillation method for determining the tryptophan content of proteins has been developed using ^{14}C -3-DT.

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INTRODUCTION

Interest in many related fields of biological research has been directed toward the characterization of receptor proteins. The binding of most biological ligands to their specific receptors involves a number of favorable interactions which together comprise a recognition process. The role of a receptor protein is dependent upon recognition and binding of a specific ligand. Thus an understanding of protein function is intimately related to the characterization of ligand binding sites irrespective of whether the binding site is the active site of an enzyme, the antigen combining region of an antibody, the hormone binding site of a receptor, the substrate binding site of a transport system, or one of the numerous nucleic acid-protein interactions which enable the expression of genetic information.

The total binding free energy of most biological interactions is usually sufficient to allow some latitude in modifying the natural ligand while retaining specificity. Thus a number of drugs and inhibitors have been designed to complement biological systems with good success. Affinity labeling methods, wherein a chemically reactive group is attached to a natural ligand or analog, make use of this latitude in specificity to achieve receptor site binding followed by covalent attachment. These methods have been quite successful in probing the ligand-macromolecule interactions which underlie receptor or recognition processes.

Unfortunately, classical affinity methods, relying on nucleophilic or electrophilic reaction at the binding site, have also been unsuccessful in many instances. Ligand binding sites on hormone receptors,

antibodies and allosteric sites of enzymes often do not contain the required hyper-reactive residues necessary to affect labeling. Additionally, solvolysis of the label, nonspecific labeling, and target site damage caused by imposed conditions of pH or temperature have limited the utility of conventional alkylating affinity labels. "Trojan Horse" labels, whose chemically labile groups are extremely reactive but remain masked until after reaching a desired target site, can alleviate many of these problems. Present designs of such labels may be classified into two categories: the so-called k_{cat} or "suicide substrate" reagents (Rando, 1974, 1975; Abeles & Maycock, 1976) and photoaffinity labels. The present treatise will be concerned with the latter class of compounds (for reviews see Creed, 1974; Bayley & Knowles, 1977; Darfler & Tometsko, 1978).

Photoaffinity Labels

In the photoaffinity labeling technique, a natural ligand or analog is modified to include a chemically inert but photolabile group. After binding to the designated target has occurred, the system is irradiated and the labile species is converted to a highly reactive radical intermediate which reacts rapidly and nonspecifically, yielding covalent attachment. Photoaffinity labels were first introduced into protein chemistry by F.H. Westheimer and his colleagues in 1962. Using p-nitrophenyl diazo [14 C]acetate, their first study demonstrated inactivation of chymotrypsin by first forming a monodiazoacetyl acyl-enzyme intermediate and subsequently photolysing this intermediate at > 350 nm to generate a carbene which covalently attached to the protein (Singh et al., 1962). In a later study, it was shown that approximately 3% of the

incorporated label had attached via insertion of the carbene into alanyl C-H bonds (Schafer et al., 1966), demonstrating for the first time that an aliphatic residue in a protein could be modified. However, the major attachment product was O- ^{14}C -carboxymethylserine, resulting from a Wolff rearrangement to a ketene followed by nucleophilic attack at serine. The tendency to rearrange to less reactive species has limited the use of α -diazooacyl species in most photoaffinity labeling applications.

In contrast to diazoacyl compounds, aryl azides are nearly inert toward proteins, and can be activated at wavelengths which do not overlap the absorption region of proteins. Further, the photogenerated nitrene intermediate is less susceptible to rearrangement than is a carbene (Knowles, 1972). Aryl azides were first applied as photoaffinity labels in a study of the hapten binding site of antibodies elicited against bovine γ -globulin derivatized at lysyl residues with the 4-azido-2-nitro-phenyl group. Photolysis of the antibody-hapten complex resulted in the covalent incorporation of 1.1-1.3 moles of reagent per mole of antibody with concomittant blockage of the hapten binding site. Chromatographic separation of the peptide chains showed that the radioactive label was incorporated into the heavy and light chains in the ratio 3.5:1. Product analysis indicated selective modification of a cysteinyl and an alanyl residue (Fleet et al., 1969, 1972).

Although aryl azides and α -diazo carbonyls are by far the most commonly used photolabels at this time, a number of other photosensitive moieties are also being employed in some studies. These include carbene precursors such as diazirines (Knowles, 1972; Bayley and Knowles, 1978B)

and α -fluorodiazoo compounds (Chowdhury et al., 1976), ketones prone to triplet excitation such as α,β -unsaturated steroid ketones (Martyr & Benisek, 1973), halo ketones prone to radical dissociation such as the β -adrenergic blocker 2-(2-hydroxy-3-isopropylaminopropoxy)-N-iodobenzene (Cysyk & Prosoff, 1972), nitro aromatics such as p-nitrophenylalanine (Escher & Schwyzer, 1974, 1975; Escher, 1970), and aryl diazonium compounds such as diazotized sulfanilic acid (Tometsko et al., 1978). The versatility of photoaffinity labels as probes of complex biological structures is illustrated by recent studies on estrogen receptors (Katzenellenbogen et al., 1977), adenosine 3':5'-cyclic monophosphate receptors (Haley, 1975), acyl-coenzyme A:glycine N-acetyltransferase (Lau et al., 1977), the anion transport system of the intact RBC (Cabantchik et al., 1976), ribosomes (Cooperman et al., 1975; Schwartz et al., 1975), and the post-synaptic receptor for acetylcholine (Witzemann & Raftery, 1977).

Mechanism of Photoaffinity Labeling

In order to attain new information about a biological target of interest, an understanding of the capabilities and limitations of the photoaffinity labeling method is required. Of the various molecules of biological interest which can be studied, soluble enzymes provide the best targets for evaluation of the mechanism of photoaffinity labeling. Using enzymes as targets, the influence of irradiation alone on the biological activity can be controlled, reversible binding can be measured before photolysis, labeling which results in inactivation can be distinguished from nonspecific labeling, and the reactions of the probe and its target can be examined spectrally.

The reactions which need to be considered in a discussion of photoaffinity labeling are shown schematically in Figure 1. In this figure, E represents the target enzyme; L, the unphotolysed photoreactive ligand (or inhibitor); L^a , the photoactivated highly-reactive intermediate; L^P , the product of the photoreaction resulting from reaction with solvent or internal rearrangement; $E-L^P$, the covalent complex of inhibitor and enzyme which results in inactivation; and $E-L^n$, the covalent complex resulting from nonspecific reaction of the photoactivated ligand with enzyme (this may or may not influence enzymatic activity). Dots represent affinity interactions which are not covalent. The processes of inter-conversion are: 1, reversible (dark) inhibitor binding; 2, the solution rate of probe photolysis; 3, the rate of reaction of the reactive intermediate with solvent (or internal rearrangement to non-reactive products); 4, the rate of photolysis of the enzyme-bound probe (the fact that this may differ from 2 will be discussed in Chapter II); 5, the rate of covalent attachment of the reactive intermediate; 6, the rate of unproductive reaction which does not yield covalent attachment; 7, reversible photoproduct binding; 8, rate of nonspecific attachment of the reactive intermediate to the enzyme without an affinity interaction; and 9, reversible binding of the photogenerated intermediate.

The desired route for photoaffinity labeling is reversible binding (1) followed by light activation (4) and covalent attachment which results in inactivation of the assayed function (5). The system should be unaffected by irradiation in the absence of the photoreactive ligand. A number of the processes shown in Figure 1 can be dissected out and

Figure 1: A schematic representation of the reactions involved in photoaffinity labeling.

characterized individually. The reversible affinity of the photoreactive ligand in the dark (1) should be determined in order to assert that the ligand interacts with its intended binding site. Competitive binding experiments using natural substrates can provide a measurement of affinity and specificity. Similar experiments using the pre-photolysed ligand provide a measure of process 7. In instances where the predominating photoproduct has higher affinity than the photoreactive ligand, photoproduct inhibition can severely limit the photoaffinity labeling/inactivation process. In a photoaffinity labeling study of phosphofructokinase using a derivative of cyclic AMP, continuous replacement of photolyzed material with fresh reagent was required to achieve complete modification of the cyclic AMP binding sites (Cooperman & Brunswick, 1973). Little is known about process 9 in any system, but investigators should be aware that the affinity of the activated intermediate for the binding site may be quite different from that of its precursor or photoproducts. This may cause dissociation of the complex, limiting labeling efficiency, or may promote a "pseudo-affinity labeling" sequence: 2,9,5.

It has been suggested that introduction of diradical scavengers into the system could distinguish between true photoaffinity labeling (1,4,5) and "pseudo-affinity labeling" (2,9,5) (Ruoho et al., 1973) or nonspecific labeling (2,8) (Bayley & Knowles, 1977, 1978A) by destroying the reactive intermediates (L^a) which are generated in solution. Reagents such as p-aminobenzoic acid, dithiothreitol, hydroquinone, and soluble non-target protein have been suggested as scavengers (Bayley & Knowles, 1977). Subsequently, it has been shown that dithiols may reduce

photoreactive arylazides to non-photosensitive amines (Staros et al., 1978), and similarly that hydroquinone may reduce nitroazidophenyl derivatives (DeTraglia & Tometsko, unpublished results). Thus a great deal of caution must be exercised in interpreting "scavenging" results. It is obvious that any condition which results in destruction of the photoreactive ligand before photolysis will prevent labeling and inactivation of the target.

As with conventional affinity labeling by nucleophiles or electrophiles, success of a photoaffinity labeling experiment requires the proper orientation of the reactive intermediate relative to available attachment points in its binding site. If the reactive intermediate is oriented toward solvent, pathway 6 will be preferred over 5. Furthermore, if the rate of attachment, 5, is slower than the dissociative equilibrium, 9, then labeling efficiency will be low. Precise information regarding the lifetimes and reaction rates of nitrene, carbene and other radical species in aqueous media is as yet unavailable in the literature.

The lifetime of the reactive intermediates generated by photolysis is to a great extent determined by the spin state of the diradical electron pair. While the photolysis of aromatic azides predominantly results in the formation of singlet nitrenes with very short reactive lifetimes (10^{-12} to 10^{-6} sec), the presence of paramagnetic molecules such as oxygen can accelerate intersystem crossing forming long-lived triplet nitrenes ($> 10^6$ sec) (Olenik et al., 1976). Photolytically generated singlet carbenes and nitrenes are capable of undergoing recombination, double bond addition, and direct insertion reactions, permitting rapid incorporation into C-H, C-O, C-N, N-H or S-H bonds (Glichrist & Rees, 1969). Typical rates for

the reactions of diphenylcarbene, for example, are: $5 \times 10^9 \text{ M}^{-1} \text{ sec}^{-1}$ for recombination; $\sim 10^7 \text{ M}^{-1} \text{ sec}^{-1}$ for insertion into the C-O bond of methanol; and $5 \times 10^{-5} \text{ M}^{-1} \text{ sec}^{-1}$ for addition across the double bond of styrene (Turro, 1979). In contrast, triplet carbene and nitrene species cannot undergo concerted addition or insertion but rather react via hydrogen abstraction and radical recombination routes. In a study of the photolysis of several aryl azides in organic solvents, Reiser and co-workers (1968) found that hydrogen abstraction by an aromatic nitrene is a comparatively slow process, corresponding to a life-time of $2 \times 10^{-4} \text{ sec}$ (in ethanol) to 2 sec (in hard polymeric matrices). Precautions to limit the production of long-lived triplet species may diminish undesired reactions such as non-specific labeling. Singlet sensitizers such as triphenylene and naphthalene, which function to populate the azide excited singlet state by singlet energy transfer, may prove useful in this regard. In a study of the photolysis of 2-azidobiphenyls, pyrene was used in a unique sense; it functioned as a singlet sensitizer and also served as a quencher for the excited triplet azide (Swenton et al., 1970).

Purpose

The research reported in this treatise was undertaken for two primary reasons. First, it was desired to produce and characterize photoaffinity analogs of lysyl and arginyl cationic amino acid side chains. These would be of utility in probing the substrate binding mechanisms of several trypsin-like proteases of current interest, including thrombin kallikrein (Wong & Shaw, 1976), clostripain, and acrosin (Parrish et al., 1978). Preliminary observations in characterizing two photosensitive

benzamidine derivatives as affinity labels for trypsin revealed that the pH dependence of photoinactivation could provide a sensitive means of detecting protein conformational distortions. Thus the second thrust in this work has been to determine whether the pH dependence of photoaffinity labeling reflects accurately the binding site affinity sensed by the probe or whether the pH dependence of covalent attachment is the prime determinant. A demonstration that the pH dependence of photoinactivation is controlled by the pH dependence of pre-covalent binding would suggest that this technique could be useful in determining the pK_a 's of critical residues in the probe's binding site. This approach would lend itself to studies of functional sites such as enzyme binding sites as well as to non-catalytic targets such as binding sites on enzyme zymogens, allosteric effectors, and hormone receptors.

Chapter I presents the characterization of meta- and para-azido-benzamidine as photoaffinity labels for trypsin. In this chapter, the synthesis, physical characterization and inhibitory properties of these compounds in both dark and photolytic studies are detailed. In addition this chapter presents the pH dependence of azidobenzamidine photoinactivation. Chapter II continues the discussion of the relationship between binding site affinity and photoinactivation efficiency. Included are discussions of the relationships between photolysis rate, binding site affinity, covalent attachment rate, and the level of photoinactivation obtained. The pH dependence of meta-azidobenzamidine photoinactivation of trypsin is compared with the pH dependence of the probe's equilibrium binding affinity and with known conformational transitions in the enzyme.

During the course of these experiments, we became aware that the diazonium compounds synthesized as precursors to aryl azides are themselves photosensitive, and can often be used as photoaffinity inhibitors (Tometsko et al., 1978). Chapter III presents an account of an interesting dark reaction of a diazonium compound which was originally contrived as a photoaffinity label for the tryptic substrate recognition site.

The subsequent portion of this introduction provides a review of tryptic enzymology pertinent to these photoaffinity labeling studies.

Trypsin

The literature on trypsin-like enzymes encompasses a group of serine proteases ranging in molecular size from 20,000 to 25,000 daltons which preferentially catalyze the hydrolysis of ester or peptide bonds involving the carboxyl group of arginine or lysine. The present work deals exclusively with studies of bovine pancreatic trypsin, as the most detailed mechanistic data has been amassed for this enzyme. In addition, advances toward complete analysis of the crystal structure (Kossiakoff et al., 1977; Stroud et al., 1974; Fehlhammer & Bode, 1975; Bode & Schwager, 1975) make trypsin an ideal enzyme for studying structure-function relations. Native β -trypsin is composed of a single chain of 223 amino acids. Activation of the enzyme from its zymogen is accomplished by splitting off an N-terminal hexapeptide at the LYS-15 - ILE-16 bond¹ autolytically or via the action of enterokinase. Subsequent cleavage of β -trypsin at the LYS-145 - SER-146

¹The presently accepted numbering system for trypsin is based on the sequence of chymotrypsinogen A and is organized such that sequence homologies in all of the serine proteases are numbered identically. A complete listing may be found in the review by Hartley & Shotton (1970).

bond leads to α -trypsin, a two chain active structure held together by disulfide bonds. Cleavage of α -trypsin at the LYS-188 - ASP-189 bond yields another active form ψ -trypsin. Commercial preparations contain some inactive protein and a mixture of active forms in an approximate ratio 7:2:1 of β , α , and ψ respectively (Fehlhammer & Bode, 1975). The active species are not kinetically distinguishable.

A great deal of primary structural similarity is found between trypsin and the other serine proteases. For example, chymotrypsin A and trypsin are homologous in 53% of their amino acid sequence.² This homology extends throughout the mammalian serine protease family. Chymotrypsins A and B, trypsin, elastase and thrombin are all chemically similar in 25% of their amino acid sequence, only 22% of the positions show no homology (Hartley & Shotton, 1970). Gross similarity in the tertiary structures has been confirmed by X-ray analysis (Krieger et al., 1974).

Homology in serine protease structure has apparently resulted in analogous catalytic mechanisms. Acylation of SER-195 by substrate, a reaction assisted by a charge relay complex composed of ASP-102 and HIS-57, proceeds identically in the chymotrypsins and trypsin (Keil, 1970; Inagami, 1972). Not only is the charge relay system identical, but many groups in its periphery are quite similar. Thus, it has been concluded that chymotryptic and tryptic hydrolysis proceed by the same mechanism.

Trypsin possesses a very narrow specificity for lysyl and arginyl esters and peptides, uncommon among other serine proteases (Keil, 1970).

²Homology is defined as ARG = LYS, ASP = ASN, GLU = GLN, ASP = GLU, ASN = GLN, SER = THR, VAL = ILE, ILE = LEU, TYR = PHE = TRP, in addition to identities. Common deletions are discounted.

Two interactions which contribute to the mechanism can be defined by inhibitor studies: 1) specific binding of the substrate (or inhibitor) side chain by residues in the recognition subsite and 2) conformational effects upon binding the side chain which activate the catalytic site.

The subsite of trypsin which recognizes and binds substrates and inhibitors consists of two areas, a negatively charged moiety and a hydrophobic area (Inagami, 1972). Identification of a carboxyl group, ASP-189, at the inhibitor binding site has been reported based upon chemical modification of the carbodiimide-activated residue with ^{14}C -glycinamide (Ely & Inagami, 1971) and confirmed by X-ray diffraction analysis (Stroud et al., 1974). The negatively charged carboxyl interacts electrostatically with the ammonium or guanidinium group of tryptic substrates and inhibitors including benzamidine. ASP-189 occurs in a sequence which is not homologous with the corresponding sequence of chymotrypsin. This sequence (ALA-183 - SER-190) is two amino acids longer in trypsin than in chymotrypsin, and may represent an evolutionary insertion to create a new enzyme by addition of the anionic binding site.

The hydrophobic region is homologous with that of chymotrypsin and is topologically located between ASP-189 and the catalytic charge relay complex ASP-102 - HIS-57 - SER-195. Crystallographic results indicate that when benzamidine is bound, it is sandwiched in a hydrophobic cleft between the peptide planes of residues 214 to 216 and 190 to 191 (Krieger et al., 1974). Although responsible for recognition and binding of the aromatic substrates of chymotrypsin, the utility of this homologous region in trypsin is somewhat ambiguous, as it retains high specificity

for aromatic rings which are not associated with tryptic substrates.

Table I compiles the results of several studies which report thermodynamic parameters for the binding of inhibitors to trypsin. The following observations can be drawn from these data:

a) Regardless of associated hydrocarbon substituents, the ammonium or guanidinium groups of inhibitors consistently contribute -10 kcal/mole or -4.5 kcal/mole, respectively, to the binding free energy. This contribution is reflected in the enthalphy (ΔH°) of binding and is interpreted as the result of salt-link formation with ASP-189 (Inagami, 1972).

b) Addition of methylene units in the series methyl through n-butyl amine or methyl through n-propyl guanidine yields a consistent increase in binding free energy. This increase, not reflected in ΔH° , must be a contribution from favorable binding entropy.

c) Inhibitors longer than n-butylamine or n-propylguanidine are less tightly bound, probably due to steric exclusion from the binding site. This is demonstrated by the decreased affinities of n-hexylamine and n-butyl-guanidine.

d) Planar aromatic inhibitors bearing a charged substituent are very tightly bound. This effect is seen with benzylamine, phenylguanidine and the aromatic amidine series.

Based upon these observations it is obvious that electrostatic interaction with ASP-189 dominates the binding free energy of inhibitors (and substrates) with trypsin. Interpreting the entropically favorable addition of methylene groups as indicative of hydrophobic bonding (point b above), several workers have suggested that the hydrophobic cleft also plays a rôle in binding (Mares-Guia & Shaw, 1965; Inagami & York, 1968).

Table I

THERMODYNAMICS OF THE FORMATION OF ENZYME-INHIBITOR COMPLEXES FOR
COMPETITIVE INHIBITORS OF TRYPSIN

Inhibitor	ΔK_I (mM)	ΔG° (kcal/mole)	H° ¹	Reference
Amine ²				
methyl-	260	-0.8	-13	Inagami & York, 1968
ethyl	62	-1.6	-11	Inagami, 1964
n-propyl-	8.7	-2.8	-10	"
n-butyl-	1.7	-3.8	-11	"
n-hexyl-	12	-2.6	-10	"
benzyl-	0.4	-4.6	-10	"
Guanidine ³				
methyl-	4.40	-3.1	-4.4	Mares-Guia, 1968
ethyl-	1.4	-3.7	-4.8	Inagami & York, 1968
n-propyl-	0.53	-4.3	-4.5	"
n-butyl-	1.3	-3.8	-4.5	"
cyclohexyl-	4.4	-3.2	-4.3	"
phenyl-	0.0725	-5.5	-4.8	"
Amidine ⁴				
β -naph	0.0146	-6.4	-	Mares-Guia, 1968
benz-	0.0166	-6.3	-	"
p-aminobenz-	0.00825	-6.7	-	Mares-Guia & Shaw, 1965
m-azidobenz-	0.040	-6.0	-	DeTraglia et al., 1978
p-azidobenz-	0.60	-4.3	-	"
Benzene ²	1000	0.0	-	Inagami, 1964

¹Determined from the temperature dependence of K_I .

²pH 6.6, 25° C.

³pH 8, 25° C.

⁴pH 8.15, 15° C; except azides, pH 6.2, 22° C.

If tryptic specificity was only conferred by the relative binding affinities of substrates, then we would expect the enzymatic catalysis rate of all substrates at saturation to be identical. This expectation results from the hypothesis that the enzyme's catalytic site is complete before substrate binding: the "key-lock" hypothesis proposed by Emil Fisher (1894). In trypsin, this seems not to be the case. As shown in Table II, the rate of hydrolysis of N-acetylglycine ethyl ester is 2000-fold slower than that of N- α -acetyl-L-lysine methyl ester when the former is at saturating concentrations. A better model based on the so-called "induced-fit" hypothesis of Koshland (1959, 1963), suggests a mechanism in which binding of the basic side chain causes a conformational transition which activates the catalytic site. Lacking an allosteric activator, non-specific substrates such as N-acetylglycine ethyl ester and p-nitrophenyl acetate are hydrolysed at a much slower rate. The process of conformational activation by the substrate side chain itself is believed to be of prime importance (together with binding in the recognition site) in determining the high specificity of trypsin.

Consistent with the substrate activation model, Inagami and York (1968) have found that the binding of small alkylamines and guanidines at the specificity site enhances the rate of tryptic hydrolysis of the so-called non-specific substrates (which interact only with the catalytic site). Methylguanidine and ethylamine, inhibitors of specific substrate hydrolysis (see Table I), cause up to a 12-fold increase in the rate of N-acetylglycine ethyl ester hydrolysis. Butylamine and larger inhibitors (Mares-Guia, 1968) decrease the rates of specific and non-specific

Table II

KINETIC PARAMETERS FOR THE TRYPTIC HYDROLYSIS OF VARIOUS SYNTHETIC ESTER SUBSTRATES. Adopted from Inagomi (1972)

Substrate	K_m (mM)	k (cat) (sec^{-1})
Specific substrates		
N- α -acetyl-L-lysine methyl ester	0.25	52
N- α -tosyl-L-arginine methyl ester	0.013	60
Non-specific Substrates		
N-acetylglycine ethyl ester	800	0.028
p-nitrophenyl acetate	21	0.013

substrate hydrolysis, probably by occluding the catalytic site. In conjunction with the binding of methylguanidine, an increase in the reactivity of HIS-57 toward alkylation with iodoacetamide has been observed (Inagamo & Hatano, 1969). Thus, it seems that binding to the specificity site in trypsin induces a conformational change which somehow alters the reactivity of the catalytic histidyl imidazole. This alteration has recently been shown to primarily affect the acylation step in catalysis, with little effect on the deacylation reaction (Vajda & Szabo, 1978).

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CHAPTER I

Characterization of Azidobenzamidines as Photoaffinity Labels for Trypsin

ABSTRACT

Meta- and para-azidobenzamidine have been prepared and evaluated as photoaffinity labels. The compounds inhibit trypsin reversibly in the dark and are competitive with substrate binding. Upon photolysis, irreversible noncompetitive inhibition is observed and is dependent upon concentration, photolysis time, and pH. Specificity of the probes is indicated by experiments in which p-tosyl-L-arginine methyl ester, a trypsin substrate, is used to protect against photoinactivation. Maximum inactivation of trypsin is achieved at pH 6.2 using either azidobenzamidine derivative. Evaluation of the pH dependence of photoaffinity labeling may provide a sensitive tool for probing conformational changes in inhibitor binding sites. These studies provide a basis for the use of azido-benzamidines as photoaffinity analogs of lysine and arginine side chains.

INTRODUCTION

Benzamidine is a potent reversible inhibitor of trypsin (EC 3.4.21.4) whose protonated amidino group interacts electrostatically with the anionic carboxyl moiety of Asp 189 in the tryptic substrate recognition site (Mares-Guia & Shaw, 1965). Since its initial characterization, a number of compounds derived from benzamidine have been developed as reversible (Tidwell et al., 1976) as well as irreversible (Wong & Shaw, 1976) trypsin inhibitors. Interest is sustained by their efficacy in inhibition of a number of arginine- and lysine-specific proteases, lending possible biochemical and therapeutic value. Among the other common target enzymes are thrombin, plasmin and kallikrein. Recent evidence has also demonstrated the utility of benzamidine in stabilization of the zymogen, proacrosin, during its isolation from bull sperm (Zahler & Polakoski, 1977).

Currently, our laboratory has been involved in the development of photosensitive aryl-azides as affinity labels for enzymes and cellular transport systems (Darfler & Tometsko, 1978; Tometsko & Turula, 1976 A,B; Senior and Tometsko, 1976; Hare et al., 1976). These compounds can be directed by ring substitution to give reversible binding to specific targets. Upon photolysis with near-UV light, the azide moiety decomposes to a highly reactive nitrene, capable of covalent attachment by C-H bond insertion or addition (Knowles, 1972). Consequently, we are interested in azido benzamidine derivatives as photoaffinity labels both from the standpoint of their potency against tryptic proteases and as lysyl or arginyl analogs to probe amino acid transport processes.

In this paper we report the synthesis of meta-azidobenzamidine (m-ABA)¹, I and para-azidobenzamidine (p-ABA), II, and their kinetic analysis as photoaffinity inhibitors of trypsin.

¹ Abbreviations used are: meta-azidobenzamidine, m-ABA; para-azidobenzamidine, p-ABA; p-tosyl-L-arginine methyl ester, TAME; and N- α -carbobenzoxy-L-lysine-p-nitrophenyl ester, CBZLysONp.

Plate 1: Chemical structures of meta-azidobenzomaidine (I) and para-azidobenzamidine (II).

I

II

EXPERIMENTAL PROCEDURES

Materials

Bovine pancrease trypsin (twice crystallized, salt-free, lyophilized) was obtained from ICN Pharmaceuticals. Tryptic substrates N- α -carbobenzoxy-L-lysine-p-nitrophenyl ester HCl (CBZLysONp) and p-tosyl-L-arginine methyl ester HCl (TAME) were obtained from Sigma and ICN Pharmaceuticals, respectively. Azidobenzamidine precursors, m-aminobenzamidine dihydrochloride monohydrate and p-aminobenzamidine HCl, were obtained from Aldrich and Sigma, respectively. Sodium nitrite (NaNO_2) was C.P. Baker "Analyzed" grade. Sodium azide (NaN_3) was from Fisher Scientific.

Most studies were carried out using 0.1 M succinate buffer, pH 6.2, with 0.02 M Ca^{2+} included. A mixed buffer containing 0.05 M succinate and 0.05 M Tris with 0.02 M Ca^{2+} was used to obtain stable values in the range of pH 3.0 to 9.5. Final adjustments to desired pH values were made with 1 N NaOH or 1 N HCl as required.

Photolysis Conditions

Photolysis was carried out with a Model 457 Micropulser (Xenon Corporation) equipped with a Xenon flash lamp (N-752C-WC) as described previously (Tometsko & Turula, 1976A). Energy was delivered at a rate of 14 pulses per second utilizing a 6 kV potential on the lamp, located 2.5 cm from the sample. Unless otherwise stated, photolysis was for a period of 10 minutes. Samples were contained in 1.5 x 5.0 cm Pyrex petri dishes fitted with 5.0 cm diameter polystyrene Falcon petri dish covers to act as filters. This configuration provided efficient thermal equilibration in addition to a very sharp filter cutoff of wavelengths below 295 nm. The entire lamp housing and sample compartment was maintained at 5-7° C

by enclosure in a small refrigerator. Under these conditions, control enzyme preparations retain greater than 95% activity when exposed to 20-minute irradiations.

Syntheses

Compounds should be handled cautiously owing to the known instability of diazo salt intermediate products. All syntheses and handling of light-sensitive materials should be performed in subdued light.

Meta-azidobenzamidine hydrochloride (m-ABA)

Meta-aminobenzamidine hydrochloride monohydrate (700 mg) was added to concentrated hydrochloric acid (3 ml) at 0° C in a dry ice-acetone bath. Sodium nitrite (240 mg, 10% excess) was added slowly and allowed to react for 30 minutes maintaining 0° C with constant stirring. Sodium azide (240 mg, 20% excess) was then added at 0° C with constant stirring. After 30 minutes, the mixture was heated on a steam bath and sufficient concentrated HCl was added to completely solubilize the reaction products. The solution was filtered hot to remove insoluble material. Crystalline m-ABA was recovered by chilling this filtrate to 0° C. The product was recrystallized twice from HCl, then dried in-vacuo over CaCl_2 . Yield was 400 mg of white crystals which decompose before melting at 194° C. (The product rapidly discolors yellow when left exposed to room light.) m-ABA was stored frozen until used. Solution concentrations were calculated assuming a formula weight of 197.6.

Infrared analysis (KBr pellet) (Figure 1) revealed ν_{max} (cm^{-1}) 1272 vs, 1295 vs ($\nu_{\text{S}}-\text{N}_3$ azide); 1470 vs, 1600 w (ν phenyl nucleus); 1645 m, 1660 vs ($\nu \text{C}=\text{NH}$ imine); and 2110 vs ($\nu_{\text{as}}-\text{N}_3$ azide). The azide bands can

Figure 1: Infrared spectrum (KBr pellet) of m-azidobenzamidine·HCl. Assignments are given in the text. Retrace at $\sim 2100\text{ cm}^{-1}$ was recorded after exposing the pellet to 10 minutes of photolysis.

be observed to decrease in intensity upon photolysis of the pellets for 10 minutes. Proton NMR spectra (Figure 2) were obtained at 10 mg/ml in D_2O using the H-O-D resonance as a reference (positions reported as parts per million downfield from H-O-D). Spectra revealed a complex aromatic proton resonance pattern as follows (position, relative intensity):

(2.93, 0.06), (2.86, 0.30), (2.81, 0.30), (2.78, 1.00), (2.72, 0.23), (2.69, 0.35), (2.67, 0.42); consistent with a meta substitution product.

Para-azidobenzamidine sulfate (p-ABA)

Para-aminobenzamidine hydrochloride (500 mg) was added to concentrated sulfuric acid (2 ml) at $0^\circ C$ on a dry ice-acetone bath. Sodium nitrite (200 mg) and sodium azide (200 mg) were added and reacted sequentially, as described for m-ABA. The desired product precipitated in the reaction vessel and was collected by filtration and washed with cold 50% EtOH- H_2O (v/v). Crystals were hot filtered and cold recrystallized twice from 50% EtOH- H_2O , then dried in-vacuo over $CaCl_2$. Yield was 400 mg of crystals light orange in tint and with slight lustre. The product discolors black at approximately $185^\circ C$, and did not melt up to $230^\circ C$. p-ABA was stored frozen. The assumed formula weight was 210.2.

Infrared analysis (KBr pellet) (Figure 3) revealed ν_{max}^{-1} (cm⁻¹) 1295 vs 1315 vs (ν_s-N_3 azide); 1485 vs 1600 vs (ν phenyl nucleus); 1645 m, 1667 vs ($\nu C=NH$ imine); and 2110 vs, 2180 m ($\nu_{as}-N_3$ azide). The azide bands decrease in intensity upon photolysis of the pellet for 10 minutes. Proton NMR (10 mg/ml in D_2O) (Figure 4) revealed an aromatic proton resonance quartet (position, relative intensity): (3.06, 0.74), (2.97, 0.93), (2.56, 1.00), (2.48, 0.73); consistent with a para substitution product.

Figure 2: Proton NMR spectrum of m-azidobenzamidine (10 mg/ml in D₂O).
Resonance of H-O-D is used as an arbitrary reference at 0 ppm.

Figure 3: Infrared spectrum (KBr pellet) of p-azidobenzamidine·(SO₄)_{1/2}. Assignments are given in the text. Retrace at ~2150 cm⁻¹ was recorded after exposing the pellet to 10 minutes of photolysis.

p-ABA

Figure 4: Proton NMR spectrum of p-azidobenzamidine (10 mg/ml in D₂O).

Resonance of H-O-D is used as an arbitrary reference at 0 ppm.

UV Spectroscopy

Solutions of m-ABA (1.87×10^{-4} M) and p-ABA (1.81×10^{-4} M) were prepared at pH 3, 6.2, and 9 in mixed succinate-Tris buffer. Ultraviolet spectra, from a Perkin-Elmer Model 550 spectrophotometer, were obtained for the dark solutions and after various times of exposure to photolysis. The observed spectral change versus photolysis time (described in RESULTS section) was characterized by first order kinetics yielding a rate of photolysis for each photoaffinity label at each pH.

Trypsin Assay

Spectrophotometric trypsin assays were carried out using CBZLysONp as substrate.

Incubation mixtures containing trypsin and the photolabel to be tested were kept on ice until assay. Specific conditions will be described subsequently for each experiment. Tryptic activity was measured by addition of 50 μ l aliquots to 2.5 ml of succinate buffer at pH 6.2 and room temperature, followed by addition of 50 μ l of CBZLysONp stock solution (10^{-2} M in 95% acetonitrile - 5% water). Optical density change at 322 nm was measured (average of 3 determinations) and the rate of spontaneous hydrolysis of substrate was subtracted (spontaneous hydrolysis rates were obtained daily with no enzyme present in the assay and generally represent < 5% of control enzyme rates). Rates were expressed as $\Delta A/\text{min.}$ or converted to percent of control enzyme activity.

pH Profiles

Mixed succinate-Tris buffers were prepared over the range of pH 3 to 9.5. To 0.5 ml of each buffer, 0.5 ml of trypsin stock (4 mg/25 ml in

0.1 mM HCl) was added, followed by 0.1 ml of m-ABA or p-ABA stock (15 mg/ml in 0.1 mM HCl). This yielded test solutions of 3.0 μ M trypsin and 6.9 mM m-ABA or 6.5 mM p-ABA. Two aliquots of 0.3 ml were withdrawn from each test solution; one held dark at 6° C and one photolysed at $6 \pm 1^\circ$ C for 10 minutes. The remaining test solution was used to obtain an exact pH measurement. After photolysis, 0.5 ml of cold succinate buffer, pH 6.2, was added to both dark and photolysed samples. These solutions were assayed for tryptic activity and analyzed spectrally to determine the degree of probe photolysis (ΔA dark minus photolysed at 230 nm for m-ABA, 273 nm for p-ABA). Rates were expressed relative to a dark control at pH 6 with no probe present.

Concentration and Photolysis Time Profiles

Various dilutions of m-ABA and p-ABA stock solutions (15 mg/ml in 0.1 mM HCl) were prepared with 0.1 mM HCl to give intermediate stock concentrations of 0 to 35 mM. To 0.1 ml of each stock probe solution, 0.5 ml of pH 6.2 succinate buffer and 0.5 ml of trypsin stock (4 mg/25 ml in 0.1 mM HCl) were added. This yielded test solutions containing 3.0 μ M trypsin and 0 to 3.6 mM m-ABA or p-ABA. Aliquots (0.3 ml) were withdrawn from the dark test solutions and after 1 and 10 minutes of photolysis; these were combined with 0.5 ml of pH 6.2 succinate buffer and assayed for tryptic activity.

Inhibition Kinetics

Michaelis parameters were obtained from assays wherein CBZLysONp stock concentrations were varied from 10^{-2} to 10^{-3} M. Exact substrate concentrations at assay were calculated by measuring A_{270} using

$\epsilon_{270} = 8980 \text{ M}^{-1} \text{ cm}^{-1}$ for CBZLysONp. Aside from substrate stock variations, assay procedures remained as previously described. Data were treated by Lineweaver-Burke analysis.

For studies of dark interactions, test solutions of $3.0 \text{ } \mu\text{M}$ trypsin in pH 6.2 succinate buffer were prepared with probe concentrations of m-ABA = 0, 6.9, 35 mM; or p-ABA = 0, 13, 32 mM. These solutions yielded concentrations at assay of $0.06 \text{ } \mu\text{M}$ trypsin and m-ABA = 0.0, 0.13, 0.66 mM; or p-ABA = 0.0, 0.25, 0.62 mM.

For studies of photolytic interactions, test solutions of $3.0 \text{ } \mu\text{M}$ trypsin in pH 6.2 succinate buffer were prepared with probe concentrations of m-ABA = 0.0, 0.10, 0.30 mM; or p-ABA = 0.0, 0.13, 0.32 mM. These solutions were then subjected to photolysis for 10 minutes. (It is important to note that the concentrations listed for these solutions refer to conditions at photolysis.) Subsequently, 0.3 ml aliquots were withdrawn and diluted by addition of 0.5 ml of succinate buffer then assayed. (Dilution is to insure complete separation of dark from photolytic inhibition.)

Substrate Protection

TAME solutions (0 to 100 mM) were prepared in water by dilution from a stock (78 mg/2 ml in water). Test solutions of $3.0 \text{ } \mu\text{M}$ trypsin and 0.35 mM m-ABA or p-ABA were then prepared in pH 6.2 succinate buffer and incubated on ice. To 2.2 ml of trypsin-probe test solution, 0.2 ml of a TAME solution was added, a 1 ml aliquot withdrawn (dark), and the remainder was photolysed for 10 minutes. The procedure was repeated for both probes at each TAME concentration. When complete, the dark and photolysed

test solutions were warmed to room temperature to allow excess TAME to hydrolyse. The solutions were then assayed for tryptic activity versus CBZLysoNp as usual. The absorbance at 285 nm for m-ABA or 275 nm for p-ABA was recorded for each test solution as an indicator of the degree of probe photolysis (see discussion of spectral characteristics), to determine whether TAME might prevent photolysis via a filtering effect.

G-25 Chromatography

Test solutions containing trypsin (13 μ M) and m-ABA (6.9 mM) or p-ABA (6.5 mM) were prepared in pH 6.2 succinate buffer. A 200 μ l aliquot of each solution was chromatographed on a Sephadex G-25 column (5 mm (i.d.) x 65 mm; refrigerated in a crushed ice encasement), and developed in subdued light with pH 6.2 succinate buffer, collecting 5 drop fractions.

Portions of the test solutions were then photolysed for 10 minutes, and chromatographed identically. The dark and photolysed test solutions were assayed and compared for tryptic activity. Each fraction collected chromatographically was assayed for tryptic activity and for optical density at the isobestic point of the probe tested (267 nm for m-ABA, 245 nm for p-ABA; see discussion of spectral characteristics). Results were examined to check separation of non-covalently attached probe from enzyme, and the recoveries of tryptic activity from dark and photolysed samples were compared.

RESULTS

Spectral Characteristics

Examination of the absorption bands of photosensitive compounds provides valuable information regarding wavelength susceptibility, rate, and products of photolysis (Baurian et al., 1976). The UV spectrum of m-ABA dark and after 1, 3, 5, 10, 30 and 60 minutes of photolysis is shown in Figure 5. In the dark, a maximum absorption is observed at 230 nm ($\epsilon_{230} = 7860 \text{ M}^{-1} \text{ cm}^{-1}$), with a shoulder at 248 nm and a weak absorption at 290 nm. Upon photolysis, the bands at 230 and 248 nm decrease in intensity while the 290 nm band increases. An isosbestic point is present at 267 nm. Non-ideal behavior is observed at photolysis times greater than 10 minutes, but is limited to longer wavelengths. The photolysis is exponential in time and can be characterized by a rate constant of $k_m = 7.4 \pm 0.9 \times 10^{-5}$ per pulse. The spectra and photolysis rate are essentially invariant over the range of pH 3 to 9.

The UV spectrum of p-ABA dark and after 1, 3 and 10 minutes of photolysis is shown in Figure 6. A maximum is observed at 275 nm ($\epsilon_{275} = 5520 \text{ M}^{-1} \text{ cm}^{-1}$) which decays upon photolysis at a rate of $k_p = 4.2 \pm 0.5 \times 10^{-4}$ per pulse. An isosbestic point is present at 246 nm. Photolysis is essentially complete after 10 minutes, with little spectral change discernable at longer photolysis times. The spectra and photolysis rate show little change over the range of pH 3 to 9.

Comparison of photolysis rates reveals that p-ABA photolyses about 6 times as fast as m-ABA. This phenomenon may in part be a function of the wavelength cutoff (295 nm) used in photolysis, with p-ABA more

Figure 5: Ultraviolet absorption spectra of meta-azidobenzamidine (1.87×10^{-4} M). Solid lines for 0 (dark), 1,3,5 and 10 minutes of photolysis. Dashed and dotted lines represent 30 and 60 minutes, respectively. Photolysis rate (insert) is $k_m = 7.4 \pm 0.9 \times 10^{-5}$ per pulse.

Figure 6: Ultraviolet absorption spectra of para-azidobenzamidine (1.81×10^{-4} M). Solid lines for 0 (dark), 1 and 3 minutes of photolysis. Dotted line represents 10 minutes. Photolysis rate (insert) is $k_p = 4.2 \pm 0.5 \times 10^{-4}$ per pulse.

susceptible to longer wavelength radiation. The exact wavelength of maximum susceptibility for each probe remains to be determined.

Effect of pH on Photoinactivation

It is of interest to study the pH dependence of affinity label interactions in order to maximize observed effects. In the case of m-ABA and p-ABA photoaffinity labeling, where photolysis is not a pH-dependent process, it is expected that any change in the number of binding sites occupied by probe would be reflected in the degree of subsequent photoinactivation.

The pH dependence of photoinactivation of trypsin with m-ABA is shown in Figure 7A. Invariance of the dark effect with pH attests that no irreversible inactivation was caused by incubating the enzyme at extremes of pH. The greatest sensitivity to photolysis is observed at pH 6.2. A rather sharp acid cutoff for the effect centers at about pH 5.7. Half-maximal effect toward more basic values occurs at pH 8.

Figure 7B presents the pH dependence of photoinactivation by p-ABA. The pH effect is less pronounced but maximal inactivation again occurs at pH 6.2.

Influence of Concentration and Photolysis Time

The effects of m-ABA and p-ABA concentration on photoinactivation are shown in Figure 8. As expected, extending the time of photolysis or increasing probe concentration results in greater inactivation. Half maximal inactivation (50% activity) by m-ABA occurs at about 0.3 mM after 10 minutes of photolysis. Similar inactivation by p-ABA is achieved at less than 0.2 mM. The indication of greater photoinactivation by p-ABA

Figure 7: A. pH dependence of photoinactivation of trypsin by 6.9 mM m-ABA. Circles represent activities of samples incubated dark at each pH. Triangles represent the pH dependence of inactivation observed after photolysis. All assays were performed at pH 6.2. For details see text.

B. pH dependence of photoinactivation of trypsin by 6.5 mM p-ABA.

Figure 8: A. Effect of m-ABA concentration on photoinactivation of trypsin.

B. Effect of p-ABA concentration on photoinactivation of trypsin.

Solid lines represent dark control, dotted and dashed represent 1 and 10 minutes photolysis respectively. Percent activity is relative to control enzyme without probe. Trypsin concentration = 3.0 μM , pH 6.2.

at lesser concentrations is reinforced by examination of the curves for one minute photolysis (dotted lines). Factors which could lead to the observed effect are a greater affinity of p-ABA for the binding site or more efficient photolytic attachment of p-ABA (recall $k_p = 6 \times k_m$). Kinetic studies were pursued to distinguish these possibilities.

Kinetics of Dark and Photolytic Inhibition

Both m-ABA and p-ABA competitively inhibit trypsin in the dark as shown in Figures 9A & 10A. The Lineweaver-Burke plots demonstrate an increase in slope with increased probe concentration while $1/V_{\max}$ remains constant. The slopes may be characterized by the formulation $(K_m/V_{\max}) \times (1 + (I)/K_I)$ in accord with standard procedure. The competitive inhibition constant for m-ABA is found to be $K_{IC} = 4.0 \pm 0.2 \times 10^{-5}$ M, that for p-ABA is $K_{IC} = 6.0 \pm 0.1 \times 10^{-4}$ M. These data indicate a difference in binding affinity with m-ABA about 15 fold tighter than p-ABA. This finding is in contrast to the result of the concentration dependence of photoinactivation (Figure 8).

Upon photolysis, the probes are activated and irreversibly inhibit the enzyme, as shown in Figures 9B & 10B. Double reciprocal plots demonstrate the expected decrease in $V_{\max}$. That K_m remains unchanged can be demonstrated by extrapolation to common abscissa intercepts. The inhibition in each case may be characterized by classical non-competitive kinetics with $K_{Inc} = 2.7 \pm 0.1 \times 10^{-4}$ M for m-ABA and $K_{Inc} = 1.8 \pm 0.1 \times 10^{-4}$ M for p-ABA. This finding asserts the previous indications that p-ABA yields similar photoinactivation at lower concentrations than m-ABA. The non-competitive inhibition constants agree very well with concentrations at half-maximal inactivation shown in Figure 8 (0.3 mM for m-ABA, < 0.2 mM for p-ABA).

Figure 9: Double reciprocal plots for the inactivation of trypsin with m-ABA, pH 6.2.

A. Dark inhibition where substrate concentration was varied while m-ABA was held constant at: 0 ($\triangle$), 0.13 ($\circ$), and 0.66 ($\diamond$) mM during assay.

K_{IC} (competitive) = $4.0 \pm 0.2 \times 10^{-5}$ M.

B. Inhibition after 10 minutes of photolysis with m-ABA present at:

0 ($\triangle$), 0.10 ($\circ$), and 0.30 ($\diamond$) mM. K_{Inc} (non-competitive) = $2.7 \pm$

0.1×10^{-4} M. Samples were diluted before assay to minimize competitive effects of non-covalently attached probe molecules. For details see text.

Figure 10: Double reciprocal plots for the inactivation of trypsin with p-ABA, pH 6.2.

A. Dark inhibition where substrate concentration was varied while p-ABA was held constant at 0 ($\triangle$), 0.25 ($\bigcirc$), and 0.62 ($\diamond$) mM during assay. K_{Ic} (competitive) = $6.0 \pm 0.1 \times 10^{-4}$ M.

B. Inhibition after 10 minutes of photolysis with p-ABA present at 0 ($\triangle$), 0.13 ($\bigcirc$), and 0.32 ($\diamond$) mM. K_{Inc} (non-competitive) = $1.8 \pm 0.1 \times 10^{-4}$ M. Samples were diluted before assay to minimize competitive effects of non-covalently attached probe molecules. For details see text.

Taken together, results of these kinetic analyses indicate that m-ABA has a stronger affinity than p-ABA for the binding site (reflected in K_{IC}), but that p-ABA is a more effective photoinactivator (reflected in K_{Inc}). The cause for this seeming paradox must relate to the differences in photolysis rate (k_m vs k_p) or steric orientation of the corresponding nitrenes. The fact that dark kinetics are competitive with CBZLysONp is consistent with interaction of both azidobenzamidine affinity labels at the substrate recognition site. Non-competitive kinetics after photolysis are consistent with covalent attachment via a nitrene intermediate.

Photolysis in the presence of nitrene scavengers has been suggested as a test of photoaffinity labeling specificity (Ruoho et al., 1973). If photoinactivation of trypsin resulted from random insertion of solution-generated nitrenes, then photolysis with azidobenzamidines in the presence of nitrene scavengers would preserve enzymatic activity. This has been shown not to be the case. In the presence of 50 mM Tris or 25 mg/ml soluble protein (chymotrypsin), both putative nitrene scavenger (Bayley & Knowles, 1977), the level of tryptic inhibition by azidobenzamidines is unaffected. Thus tryptic photoinactivation must proceed via covalent attachment of the bound probe.

Substrate Protection

As a further test for azidobenzamidine interaction at the substrate recognition site in trypsin, protection experiments with TAME were undertaken. TAME is a common substrate often used to assay tryptic activity, and does not itself have appreciable UV absorption above 280 nm. This

credential is necessary to prevent protection from photoinactivation via a filtering effect (and precludes use of CBZLysONp or the azidobenzamidine precursors in this experiment). The degree of probe photolysis was monitored for each TAME concentration used in the protection experiments and found to be constant ($\Delta A_{285} = 0.535 \pm 0.058$ for m-ABA photolysis, $\Delta A_{275} = 1.485 \pm 0.055$ for p-ABA photolysis).

Results of the substrate protection experiment are shown in Figure 11. TAME effectively competes for occupation of the azidobenzamidine binding sites and in so doing prevents photoinactivation. The effect is more pronounced for p-ABA, consistent with the previous finding that p-ABA has less binding affinity than m-ABA. TAME at 8.6 mM completely protects against p-ABA photoinactivation.

Chromatography

Molecular exclusion chromatography on G-25 Sephadex was used to separate probe molecules from trypsin. If covalent attachment is the mechanism responsible for non-competitive photoinactivation, then enzymatic activity of photolysed solutions should be recovered when probe is removed. As shown in Figure 12, we have been able to completely separate trypsin from m-ABA, p-ABA and their respective photoproducts on a small G-25 Sephadex column. The low specific activity of photolysed samples was not found to increase significantly after chromatography. Figure 12A depicts an experiment with a photolysed m-ABA solution originally possessing 11% activity relative to dark control. Chromatographic recovery was 18% activity relative to dark control. Similarly for p-ABA, in Figure 12B, an original activity of 20% of dark control compared well with 30% activity

Figure 11: Substrate protection against photoinactivation by 0.35 mM m-ABA (---·) and 0.35 mM p-ABA (----). Solid line represents activity of dark controls at each TAME concentration.

Figure 12: Chromatographic separation of azidobenzamidines and solution photoproducts from trypsin. Fractions were assayed for tryptic activity (scaled on the left axis) and probe absorbance at an isosbestic wavelength (scaled on the right axis).

A. Experiment using m-ABA. Specific activity of photolysed sample (----) remains low (18%) relative to dark control (—) after chromatography.

B. Experiment using p-ABA. Recovered specific activity of photolysed sample (----) is 30% of dark control (—).

recovered chromatographically. These results indicate that photolytic inactivation is indeed irreversible and most probably the result of covalent attachment.

DISCUSSION

The purpose of this work has been to prepare and characterize meta- and para-azidobenzamidines as photoaffinity labels. We have chosen to evaluate their action against trypsin for three reasons. First, our previous experience has indicated that certain enzymatic systems provide relatively non-complex and easily assayed targets for specific and non-specific probe binding (Darfler & Tometsko, 1978; Tometsko & Turula, 1976A,B). Second, we desired to compare these results with those obtained using other amidine derivatives. Finally, competition for the substrate recognition site can be used as a measure of structural similarity between inhibitors and the side chains of arginine or lysine (Mares-Guia & Shaw, 1965). Since we intend to probe other biological targets of similar specificity, a demonstrable effect on trypsin is a logical first step toward designing affinity labels for these targets.

Ideally, photoaffinity labeling requires specific and reversible interaction with a binding site in the dark, followed by covalent attachment upon photolysis (Knowles, 1972). We have demonstrated reversible dark interactions, photolytic activation characteristics, and irreversible photolytic inhibition of trypsin with m-ABA and p-ABA. Thus m-ABA and p-ABA appear to have the desired characteristics for use as photoaffinity labels.

Dark inhibition studies (Figures 9A & 10A) and substrate protection experiments (Figure 11) provide consistent results indicating: 1) that m-ABA and p-ABA are competitive and reversible inhibitors of trypsin in the dark, 2) that azidobenzamidines and the tryptic substrate TAME are in

direct competition for the same site, and 3) that m-ABA has a stronger affinity for the binding site than p-ABA. Results obtained by other investigators using other amidine derivatives show similar competitive binding affinities (K_I benzamidine = 1.84×10^{-5} M, K_I p-aminobenzamidine = 8.26×10^{-6} M, pH 8.15, 15° C, Mares-Guia & Shaw, 1965), and have been interpreted as interaction in the substrate recognition site. Thus we are confident that our results for azidobenzamidines (K_{Ic} = 4.0×10^{-5} M for m-ABA, K_{Ic} = 6.0×10^{-4} M for p-ABA) also represent binding to the recognition site. The difference in affinity between m-ABA and p-ABA is likely related to subtle steric requirements of the site.

Photolytic inactivation is irreversible as demonstrated by two lines of evidence. First, Lineweaver-Burke plots (Figures 9B & 10B) show classical non-competitive inhibition (K_{Inc} = 2.7×10^{-4} M for m-ABA, K_{Inc} = 1.8×10^{-4} M for p-ABA). The inhibition reflected in a lower V_{max} , indicates that sites with probe bound have been inactivated. Second, chromatographic separation of unbound probe from enzyme (Figure 12) does not restore enzymatic activity to photolysed solutions. This provides evidence for irreversible binding, presumably covalent attachment via a nitrene intermediate. Scavenging experiments with Tris or soluble protein demonstrate that the observed enzymatic inactivation does not result from random insertion of solution-generated nitrenes. Photolytic inactivation is dependent upon probe concentration and time of irradiation (Figure 8) as expected. In experiments using 2 μ M chymotrypsin and up to 5 mM azidobenzamidines, we have failed to see photolytic loss of chymotryptic activity. Thus the azidobenzamidines appear to interact selectively, mimicking tryptic substrates.

In comparing the photoinactivation by m-ABA and p-ABA (Figure 8), it has been found that p-ABA causes 50% inhibition of trypsin at lower concentrations than does m-ABA. Non-competitive inhibition constants (Figures 9B & 10B) are consistent also showing stronger inhibition by p-ABA than m-ABA. One might expect m-ABA to be a better irreversible inhibitor based on the fact that it occupies more binding sites than p-ABA in the dark at similar concentrations. The degree of irreversible inactivation is, however, strongly influenced by the relative rates of photolysis of the probes. As shown in Figures 5 & 6, p-ABA photolyses six times faster than m-ABA. This causes the "effective" concentration of activated p-ABA to exceed that of m-ABA, leading to more efficient covalent attachment. Particular attention must thus be focussed not only on affinity, but also on photolysis rate, when considering the ability of a photoaffinity probe to label its complement site. Characterization of rates of photolysis versus wavelength of irradiation could provide useful information to control photoaffinity labeling.

Another variable which influences the degree of photoinactivation is conformational accessibility of the binding site. In evaluating the pH dependence of photoinactivation of trypsin, we have found that the effects of these probes are very sensitive to changes in pH, with optimum inactivation occurring at pH 6.2. As there is no pH dependence for photolysis rate, (Figures 5 & 6) and no known pH dependence for nitrene insertion, we believe the degree of photoinactivation reflects changes in the conformation (and/or charge) of the binding site. Variations in accessibility of substrate recognition sites with pH present problems which may be uniquely resolved by photoaffinity labeling.

In conclusion, we have demonstrated specific inhibitory dark and photolytic effects of meta- and para-azidobenzamide on trypsin. This work provides evidence for the utility of azidobenzamides as probes of lysine- or arginine-specific biological targets and warrants the investigation of similar effects on other tryptic proteases or complementary cellular transport systems.

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CHAPTER II

Photoaffinity Labeling Reagents as Probes of Protein Conformation.
Labeling of Trypsin by Azidobenzamidines, 4-Fluoro-3-Nitrophenyl-
azide, and 3-Azido-1,2,4-triazole.

ABSTRACT

Certain photoaffinity labeling reagents such as 4-fluoro-3-nitro-phenylazide (FNPA), m- and p-azidobenzamidine (m- and p-ABA), and 3-azido-1,2,4-triazole (3-AT) are capable of producing varying degrees of irreversible inactivation of trypsin depending upon the pH maintained during photolysis. This phenomenon is caused by the ability of the reagents to translate the affinity of complement binding sites at any pH to a proportionate level of photoinactivation upon photolysis. The results cannot be attributed to rates of azide photolysis (nitrene generation), as these bear no pH dependence.

Maximum tryptic inhibition by m- and p-ABA and 3-AT occurs in the range of pH 6-7 and is prevented in the presence of the protecting substrate TAME. That the degree of photoinactivation observed with these inhibitors represents a measure of specific binding affinity vs pH is corroborated by comparison with: 1) the enzyme's activity profile, 2) ORD spectra, and 3) affinity vs pH data obtained for m-ABA by UV spectral titrations.

In contrast, photolytic inhibition by FNPA shows maxima at pH 3 and 9 with little inhibition at pH 6-7. This demonstrates, 1) that hydrophobic sites are revealed at pH extremes (enzyme denaturation) and 2) 'most importantly' that pH extremes are not deterrent to nitrene insertion.

These results demonstrate that under controlled conditions photoaffinity techniques may be used to probe target site conformation and enzyme function.

INTRODUCTION

The most powerful aspect of the photoaffinity labeling technique is the ability to generate through photolysis an intermediate of such high reactivity that covalent attachment to a ligand binding site can be achieved even in the absence of nucleophilic or electrophilic residues at the site. By comparison, conventional affinity labeling techniques are very limited in that requirements for nucleophile reactivity often dictate narrow ranges of pH, temperature, and concentration within which labeling experiments must be conducted. The present work will seek to demonstrate that the extreme reactivity of diradical intermediates generated during photolysis can be used to an advantage in allowing conditions such as pH to be varied during photoaffinity labeling experiments. Thus it is possible to probe the pH dependence of binding site affinity using certain photolabeling reagents and to interpret changes in affinity in terms of alterations in the conformation of protein components comprising the binding site.

In order to implement the photoaffinity labeling technique to sense protein conformation, a number of variables must be carefully considered. For instance, relationships between probe photolysis rate, binding site affinity, and photolabeling efficiency must be well understood. The interpretation of differential photoinactivation with pH as a reflection of changes in target site affinity of course relies on the assumption that neither probe photolysis rate nor nitrene attachment rate are pH dependent. Attempts to independently quantitate each of these events will be described. In order to utilize a particular photoaffinity reagent over

a broad range of pH, the stability of the photoreactive species at extremes of pH must also be documented. A final point which will be considered is that under somewhat fortuitous circumstances it may be possible to photolyse a particular probe species, such as probe bound to the target site, while not affecting the bulk of probe in solution. This phenomenon can greatly enhance the selectivity of target site labeling.

In pursuing these studies, trypsin has been chosen as a model enzyme target. In addition to providing a convenient assay system for reversible and irreversible inactivation studies, the bulk of structural data available for this enzyme has facilitated the design of effective photoaffinity inhibitors and the interpretation of results relative to documented conformational transitions (Figure 1). Inhibitor and substrate binding contacts in the active site of trypsin have been identified by X-ray crystallography (Bode & Schwager, 1975). Two types of interactions are believed to contribute the majority of binding free energy. The first is an electrostatic interaction between the carboxyl moiety of ASP-189 and the cationic side chains of amine, guanidine, and amidine ligands. A second contribution is believed to arise via contacts between the hydrophobic aliphatic or aromatic portions of inhibitors and substrates (Mares-Guia, 1968) and the peptide backbones of residues 190-191 and 214-216 (Bode & Schwager, 1975). The catalytic charge relay complex composed of ASP-102, HIS-57 and SER-195 is analogous to that in chymotrypsin.

Benzamidine has been well characterized as a competitive inhibitor of tryptic proteases and has served as the basis for the synthesis of two photoaffinity inhibitors, meta- and para-azidobenzamidine (m-ABA, p-ABA).

Figure 1: Schematic representation of the interaction of p-azidobenzamidine with the substrate binding cleft in trypsin. Reversible (DARK) binding of benzamidine derivatives is governed by an electrostatic interaction between the amidine cation and the negatively charged carboxyl of ASP-189, and hydrophobic contacts with the peptide backbone of residues 190-191 and 214-216 (Bode & Schwager, 1975). Photolytic activation of the azide moiety generates a diradical nitrene species which covalently (DeTraglia et al., 1978) attaches, probably by an insertion mechanism (Bayley & Knowles, 1977; Darfler & Tometsko, 1978). The exact site of covalent attachment has not yet been determined.

Plate 1: Chemical structures of m- and p-azidobenzamidines (m,p-ABA), 4-fluoro-3-nitrophenylazide (FNPA), and 3-azido-1,2,4-triazole (3-AT).

m,p-ABA

FNPA

3-AT

In addition to the azidobenzamidines, a more hydrophobic compound, 4-fluoro-3-nitrophenylazide (FNPA), and a five-membered-ring nitrogen heterocycle, 3-azido-1,2,4-triazole (3-AT), have been studied. 3-AT is capable of undergoing both protonation and deprotonation, varying its charge structure drastically within the normal pH range of study.

RESULTS AND DISCUSSION

Relationships Between Probe Photolysis Rate, Binding Site Affinity, and Photolabeling Efficiency

As with conventional affinity labeling, the success of a photoaffinity labeling experiment relies on a combination of binding site selectivity, reagent reactivity, and orientation. A photogenerated reagent should be generated, 1) at its intended interaction site, 2) in an orientation which facilitates covalent attachment, and most importantly 3) should be generated at wavelengths which do not damage the target system.

Studies reported here have been conducted using a photolysis system consisting of a high intensity Xenon flash lamp whose output is filtered to cutoff wavelengths below 290 nm (Tometsko & Turula, 1976; DeTraglia et al., 1978). Typically, the system is operated at 4° C with a repetition rate of 14 pulses per second. The time course of photolysis of a typical photoaffinity label can be monitored by scanning the UV spectrum of the compound as a function of photolysis time (Baurian et al., 1976) (Figure 2). Photolysis of m-ABA results in a decrease in absorbance at 230 nm with a corresponding increase at approximately 290 nm. The existence of an isobestic point at 267 nm confirms that solution photolysis proceeds with smooth conversion of the photosensitive precursor to a single predominating photoproduct. A first order rate for the solution photolysis process can be obtained by plotting the logarithm of the change in absorbance at 230 nm against time. This parameter provides a convenient means of comparing solution photolysis rates of various photoaffinity labels and under varying conditions such as pH. Photoaffinity reagents such as the azidobenzamidines,

Figure 2: Ultraviolet absorption spectrum of m-azidobenzamidine (1.87×10^{-4} M). Solid lines for 0 (DARK), 1, 3, 5 and 10 minutes of photolysis. Dashed and dotted lines represent 30 and 60 minutes, respectively. Photolysis rate (inset) is $k_p = 7.4 \pm 0.9 \times 10^{-5}$ per pulse. Synthesis of azidobenzamidines and apparatus for photolysis have been described in detail previously (DeTraglia et al., 1978).

which do not undergo protonation or deprotonation in the range of pH 3 to 10, seem to produce spectral patterns and photolysis rates which show little variation with pH (DeTraglia et al., 1978). The photolysis rate for m-ABA determined from this result is 7.4×10^{-5} per pulse, irradiating only with wavelengths above 290 nm.

A comparison of rates determined for meta- and para-azidobenzamidines reveals that the para isomer, with a photolysis rate constant of 4.2×10^{-4} per pulse, photolyzes six times more rapidly than the meta form (Table I). Inhibition studies at pH 6.2 performed using carbobenzyloxy-L-lysine nitrophenyl ester as substrate reveal that both m- and p-ABA are competitive reversible inhibitors under non-photolysing conditions. Meta-ABA has a tighter binding constant, 4×10^{-5} M, than does p-ABA, 6×10^{-4} M. Following 10 minutes of irradiation, inhibition kinetic plots for both inhibitors appear non-competitive with substrate. Interestingly, the compound which more efficiently inactivates the enzyme under photolytic conditions is p-ABA, in spite of its lower binding affinity. Thus the influence of photolysis rate and orientation of the photogenerated intermediate can have a pronounced effect on the efficiency of photoactivated attachment processes. The observed non-competitive inhibition constant of 2.7×10^{-4} M for m-ABA reflects less efficient photoinactivation than does that for p-ABA, 1.8×10^{-4} M. Substrate protection and nitrene scavenging experiments have been used to confirm that the enzyme inactivation observed with each of these compounds is subsequent to an affinity interaction.

Table I

INACTIVATION OF TRYPSIN BY AZIDOBENZAMIDINES

	Meta-ABA	Para-ABA
Photolysis rate		
k_p (per pulse)	$7.4 \pm 0.9 \times 10^{-5}$	$4.2 \pm 0.5 \times 10^{-4}$
Substrate Competitive Reversible Inhibition		
-Dark- K_{IC} (M)	$4.0 \pm 0.2 \times 10^{-5}$	$6.0 \pm 0.1 \times 10^{-4}$
Irreversible Inhibition		
-10 min. Photolysis- K_{Inc} (M)	$2.7 \pm 0.1 \times 10^{-4}$	$1.8 \pm 0.1 \times 10^{-4}$
Substrate Protection		
TAME conc for 50% protection	~ 5.5 mM	~ 3.5 mM
Nitrene Scavengers		
50 mM Tris		
25 mg/ml Chymotrypsin	No effect	No effect

Summarized from DeTraglia et al. (1978).

Ability of Photolabeling Reagents to Sense Binding Site Conformation as a Function of pH

Having noted that the rates of photolysis of such probes as the azidobenzamidines do not show a pH dependence, we have become interested in determining whether the photoaffinity labeling technique can be applied to sense structural changes in binding sites on proteins. An experiment can be performed in which a series of enzyme solutions are irradiated in the presence of a photoaffinity inhibitor at varying pH values. After readjusting all of the solutions to a common pH and assaying the enzyme activity, differential effects of photoaffinity labeling as a function of pH can be assessed. A dark control which does not undergo irradiation can be used to judge whether irreversible enzyme denaturation occurs at the pH extremes. A differential inactivation as a function of pH using a photoaffinity label whose photolysis rate is not pH dependent must then be attributed either to the pH dependence of nitrene attachment or to a conformational pH dependence of orientation or affinity at the binding site.

The pH dependence for the inactivation of trypsin by m-ABA and FNPA has been determined using the experiment described (Tometsko & Turula, 1976; DeTraglia et al., 1978) (Figure 3). The optimum acidity for inactivation by m-ABA occurs in the neutral pH region, with inactivation occurring to a lesser extent at both pH extremes. This profile is not unexpected considering that the enzyme's optimum activity is also at neutral pH (Inagami, 1972). By contrast, tryptic inactivation by FNPA is most effective at the extremes of pH (Tometsko & Turula, 1976). Again this is not inconsistent with the known hydrophobicity of FNPA. It is quite probable that

Figure 3:

Lower Frame: pH dependence for the inactivation of trypsin by 6.9 mM m-azidobenzamidine (m-ABA) (solid line) and 0.1 mM 4-fluoro-3-nitrophenyl azide (FNPA) (broken line) after 10 minutes of photolysis. Unphotolysed controls incubated at each pH retained constant activity. All samples were assayed following readjustment to pH 6.2. Data from DeTraglia et al. (1978) (m-ABA) and Tometsko & Turula (1976) (FNPA).

Upper Frame: pH dependence of conformational transitions in trypsin marked by changes in optical rotation at 436 nm in the absence (solid line) and presence of 0.5 M n-butylamine (dashed line), a competitive inhibitor (based on D'Albis & Bechet, 1967). The pH dependence for deacylation of benzoylarginine ethyl ester (BAEE), believed to represent the pK_a of the catalytic histidine residue (HIS-57), is shown in alternating dots and dashes (after Inagami, 1972). The acidic cutoff for m-ABA photoinactivation appears to correlate with protonation of HIS-57. Enzyme denaturation correlates both with loss of substrate-competitive m-ABA interaction at alkaline pH and with increased FNPA binding at pH extremes.

the interaction of trypsin with FNPA involves an affinity interaction with a hydrophobic region of the protein which is exposed at extremes of pH as the enzyme undergoes conformational denaturation. An important inference that can be drawn from these results is that pH extremes do not seem to be deterrent to the nitrene attachment process. Further studies should be undertaken to directly measure the pH dependence of the covalent attachment process. The upper frame of this figure presents the pH dependence of the enzyme's optical rotation at 436 nm both in the absence and presence of n-butylamine, a competitive inhibitor. As an indication of gross conformational changes in the enzyme, these data correlate very well with the FNPA result just described. The acidic cutoff for photoinactivation with m-ABA correlates well with the protonation of the catalytic histidine residue at the active site. The pK_a of this residue has previously been determined from the pH dependence of k_3 , the deacylation of the enzyme-substrate complex. For benzoylarginine ethyl ester, the deacylation pK_a is 6.15 (Inagami, 1972; Caplow & Jencks, 1962).

As an independent assessment of the pH dependence of m-ABA binding to trypsin, we have performed a series of equilibrium binding affinity measurements under non-photolytic conditions (Figure 4). In this experiment a trypsin solution is maintained in a spectrophotometer and is titrated by addition of precisely measured aliquots of m-ABA to both sample and reference cuvettes. As the ligand concentration is increased, a difference spectrum is recorded which saturates as all of the available binding sites become occupied. This saturation phenomenon, quantitated by measurement of the absorbance at 317 nm, can be treated by standard techniques to

Figure 4: Difference spectrum for the equilibrium titration of trypsin by m-azidobenzamidine at pH 7. Spectral curves represent 20 μM trypsin in the presence of 0, 62, 124, 248, 369 and 490 μM m-ABA (1-6 respectively). m-ABA titrant was added to both sample and reference cuvettes. Buffer was 50 mM sodium succinate, 50 mM Tris and 20 mM CaCl_2 , adjusted to the desired pH with concentrated HCl. Titrations were conducted at room temperature (22°).

Spectral saturation curves were used to compute binding affinity constants by the method of Rose & Drago (1959). See the Appendix. At pH 7.0, $K_d = 1.30 \times 10^{-4}$ M.

determine an equilibrium binding constant (Rose & Drago, 1959; see the Appendix). By repeating this experiment across a broad range of pH, a profile of equilibrium affinity versus pH can be constructed (Figure 5). This series of experiments confirms that the optimum pH for the interaction of m-ABA with trypsin is in the range of pH 6 to 7. Immediately below pH 6, and in the range of pH 7.5 to 9, the binding affinity measured by equilibrium titration decreases appreciably. These data are in good correspondence with the results of the photoinactivation experiments described previously. Thus it seems quite likely that the pH dependence of photoaffinity inactivation can be interpreted as an indication of binding site conformation.

A similar series of titration experiments can be performed to determine the pH dependence of the binding of β -naphthamidine to trypsin (Figure 7). As shown in this figure, the titration of trypsin by β -naphthamidine produces a difference spectrum (Mares-Guia, 1968) with a maximum at 250 nm, and isobestic points at 244 and 261 nm (Figure 6). Quite unlike the interaction of m-ABA, the binding of β -naphthamidine does not decrease in affinity at alkaline pH. β -Naphthamidine is a more bulky and hydrophobic inhibitor than is m-ABA, but binds roughly ten-fold tighter than m-ABA even at neutral pH. These data suggest that a steric interaction of the azide substituent on m-ABA may account for its decreased affinity relative to β -naphthamidine and that this steric interaction may become more critical at alkaline pH. It is noteworthy that the presence of Ca^{2+} in the equilibrium titration increases the affinity of β -naphthamidine binding.

Figure 5: pH profile for the binding of m-azidobenzamidine to trypsin determined by equilibrium spectral titrations (see Figure 4). The affinity dependence correlates well with the level of inactivation attained at each pH in photoaffinity inhibition experiments (Figure 3).

Figure 6: Difference spectrum for the equilibrium titration of trypsin by β -naphthamidine at pH 6.5. Spectral curves represent 20 μ M trypsin in the presence of 0, 6, 12, 19, 25, 31, 37 and 49 μ M β -naphthamidine (1-8, respectively) against a reference without enzyme. Buffer was 50 mM sodium succinate, 50 mM Tris (20 mM Ca^{+2} added in some experiments, see Figure 7). Titrations were conducted at room temperature (22°). Binding constant computed from saturation curves at pH 6.5 is $K_d = 8.00 \times 10^{-6}$ M. β -naphthamidine was prepared by reacting β -cyanonaphthalene (ICN) with anhydrous ethanol to form the imido ester; which was subsequently reacted with anhydrous ammonia to form the free unsubstituted amidine (method of Pinner & Klein, 1877).

Figure 7: pH profile for the binding of β -naphthamide to trypsin in the absence (circles) and presence of 20 mM Ca^{+2} (squares).

Aryl Azide Stability at pH Extremes

A further requirement that must be satisfied before a photoaffinity labeling experiment can be performed across a broad pH range is that the photosensitive reagent must be chemically inert and stable throughout the range of interest. Analysis of the reagents' IR, NMR and UV spectra under various conditions is often sufficient to determine whether instabilities exist. Often a compromise in temperature or time of exposure is sufficient to provide the required stability. For instance, FNPA which couples to amines by a displacement reaction when warmed at alkaline pH, can be used as a photoaffinity label at pH 10 when short exposures and low temperatures are maintained (Tometsko & Turula, 1976). Nonphotolysed controls may be used to verify inertness during dark incubations with the photolabel.

It is known that organic azides decompose rapidly after protonation by mineral acids (Patai, 1971). However, the required acidity for this reaction may be far stronger than that encountered in most biological studies (Figure 8). Studies of 3-azido-1,2,4-triazole have confirmed that in acidic media, the molecule protonates on the ring, and not on the azide (Pevzner et al., 1974). The center frame of this figure presents the infrared spectrum of uncharged 3-AT. The free azide band occurs at 2140 cm^{-1} . The upper frame, presenting the spectrum of the hydrochloride form of the molecule, indicates that the azide group is present in its free form, again at 2140 cm^{-1} . In fact, the molecule is stable when stored in 95% sulfuric acid. There are also indications in the literature that azide substituents present on electron donating ring systems may tautomerize to a less photosensitive tetrazole (Patai, 1971; Heitke & McCarthy, 1974).

Figure 8: Infrared spectra for 3-azido-1,2,4-triazole (3-AT) as a hydrochloride salt (upper spectrum), uncharged (middle spectrum), and as an anion (lower spectrum). At acid pH, the molecule protonates on the ring, not on the azide; evidenced by the unchanged frequency of the azide stretch (2140 cm^{-1}) in both uncharged and protonated forms (Mel'nikov et al., 1974). At alkaline pH, deprotonation to an anionic form is not accompanied by a tautomeric conversion of the azide to a tetrazole, again evidenced by the unchanged azide frequency at 2140 cm^{-1} . Protonation and deprotonation pK's were determined by aqueous titration with KOH (see Figure 9). Infrared spectra were recorded as KBr pellets. 3-AT was prepared by the method of Heitke and McCarty (1974).

As shown in the lower frame of this figure, the anionic azidotriazole also possesses a free azide band at 2140 cm^{-1} . The existence of a tetrazole tautomer for this molecule is therefore highly unlikely. The pK_a values shown for protonation at pH 1.68 and deprotonation at pH 8.5 have been determined by aqueous titration of 3-AT with KOH (Figure 9).

Using 3-AT as an example, one cannot overemphasize the need to fully examine the chemistry of photoaffinity labels before applying them to biological systems.

Selective Photolysis of a Desired Photoreactive Species

A phenomenon that is not often considered in studies with photoaffinity labels is that the absorption characteristics of the bound probe may differ from those of the species in free solution. Red or blue shifts in absorption due to differing environments encountered by the affinity label can alter its photolysis characteristics. In our studies of the interaction of 3-AT with trypsin, we have made some interesting observations.

The concentration dependence of tryptic photoinactivation by 3-AT at pH 6.5 using 10-minute photolyses is shown in the lower frame (Figure 10). Fifty percent inactivation is achieved at approximately 4 mM 3-AT. Take note of the fact that in the upper frame the time required to photoinactivate half of the enzyme is only about two minutes, with the maximal inactivation achieved by 10 minutes. This rapid rate of photoinactivation presents a paradox when compared with the rate of photolysis of 3-AT in solution (Figure 11). As shown in the left portion of this figure, 3-AT has an absorption maximum at 233 nm. Irradiating with a 290 nm cutoff filter, less than 5% of the compound is photolysed during a 10-minute

Figure 9: Aqueous titration of 3-azido-1,2,4-triazole with KOH. $pK_1 = 1.68$, $pK_2 = 8.50$. One millimole of 3-AT, dissolved in absolute ethanol, was converted to the hydrochloride by bubbling anhydrous HCl through the solution for 15 minutes. Subsequently, ethanol and residual HCl were removed under vacuum. After addition of 20 ml of H_2O , the sample was titrated against 0.250 M KOH.

Figure 10:

Upper Frame: Photolysis dependence for inactivation of trypsin by 15 mM 3-AT at pH 6.5 (photolysis conditions described previously). Half maximal inactivation is obtained with ~2 minutes photolysis time.

Lower Frame: Concentration dependence for inactivation by 3-AT using 10 minute photolyses. Half maximal inactivation occurs with ~4 mM 3-AT. All samples contained 0.2 mg/ml trypsin plus appropriate concentration of 3-AT in Tris-succinate buffer with 20 mM Ca^{+2} . Activity was measured by a modification of the method of Erlanger et al. (1961), using benzoyl-L-arginine-p-nitroanilide as substrate

Figure 11: Ultraviolet absorption spectrum of 3-AT (100 μ M) dark (0 min.) and after 5 and 10 minutes of photolysis using the system previously described, with a 290 nm cutoff filter. Less than 5% of the compound is photolysed in 10 minutes under these conditions. Also shown is a spectrum following one minute of unfiltered irradiation.

Inset: Absorption difference spectrum for the binary complex of trypsin with 3-AT, obtained by titration of 3-AT (100 μ M) with increasing amounts of trypsin (0-100 μ M; 1-5 respectively). The binary complex absorption, which extends to wavelengths above 290 nm, probably accounts for acceleration of the photolysis of 3-AT when it is bound to trypsin.

exposure. Thus, there seems to be no correspondence between the rate of photolysis of 3-AT and its rate of photoinactivation of trypsin. A possible explanation, however, is that the species bound to trypsin is more susceptible to photolysis than is the unbound probe. That this may in fact be the case is illustrated in the inset. A difference spectrum for the bound probe can be obtained by titration in a manner similar to that described earlier for m-ABA. In this case a 100 μ M 3-AT solution is titrated by addition of trypsin aliquots to both sample and reference. The resulting spectrum represents that of the bound probe species, and shows a second maximum at 275 nm. The absorption of this new band extends beyond 290 nm and therefore accounts for the increased photolytic susceptibility of the enzyme-bound probe seen in photoinactivation time studies.

An examination of the rate of incorporation of 14 C-labeled 3-AT into trypsin at pH 6.5 reveals that within the first 10 minutes of photolysis, the ratio of probe incorporated into enzyme approaches 1:1 (Figure 12). Thus, the species most rapidly photolysed and accounting for the enzymatic inactivation, probably binds to a discrete functionally related site on the enzyme. Recall that at pH 6.5, 3-AT is an uncharged molecule. In spite of the fact that it does not bear a cationic structure at neutral pH, our data indicate that the photoinactivation of trypsin results subsequent to interaction of 3-AT at the substrate binding site (Figure 13). In the presence of TAME, complete protection against photoinactivation can be achieved.

Considering its variable charge structure and the fact that uncharged 3-AT binds reversibly to the substrate recognition site on trypsin, it is

Figure 12: Incorporation ratio (squares) and percent activity (circles) as a function of photolysis time for photoaffinity labeling of trypsin by ^{14}C -3-AT. Within the first 10 minutes, decreasing activity corresponds directly with a single probe molecule incorporated per protomer. Irradiation and activity measurements were conducted as previously described. Radiolabeled 3-AT was prepared from 5- ^{14}C -3-amino-1,2,4-triazole (ICN) by the same procedure as the unlabeled reagent. The method for incorporation ratio determination will be described in detail elsewhere (DeTraglia et al., 1979; Chapter III of this thesis).

Figure 13: Protection of trypsin against photoinactivation by 5 mM 3-AT using p-tosyl-L-arginine methyl ester (TAME) as a competing substrate. Experimental method has been described elsewhere (DeTraglia et al., 1978).

5 mM 3-AT
10 min. PHOTOLYSIS

interesting to examine the pH profile for inactivation of the enzyme by this probe (Figure 14). This experiment is somewhat the opposite of that performed using the azidobenzamidines. Meta-azidobenzamidine retains the same charge structure throughout the pH range and may therefore be used to sense the conformation of its complementary binding site on the enzyme. In contrast, the result obtained with 3-AT suggests that the probe's charge structure dictates its inactivation profile. As the pH approaches the alkaline pK of 8.5, the probe attains a net negative charge, and does not interact with the enzyme. As the pH decreases below 6, the probe loses its ability to bind to the enzyme until the pH reaches approximately 4. Below pH 4, the concentration of protonated azidotriazole begins to approach the millimolar range. This distinctly cationic species interacts very strongly with its binding site on trypsin and is able to produce significant levels of inactivation in the pH 3 to 4 region.

Figure 14: pH dependence for the inactivation of trypsin by 15 mM 3-AT with 10 minute photolysis. Alkaline cutoff agrees with the deprotonation of 3-AT at pH 8.5. At pH values below pH 4, the increased photo-inactivation may result from protonation of 3-AT ($pK_1 = 1.68$), generating a cationic species which binds very tightly to the enzyme.

SUMMARY

These results suggest that important information regarding the conformation of complementary binding sites and the behavior of various charged states of inhibitors can be obtained from studies of the pH dependence of photoaffinity labeling experiments. Such information may assist in identifying the pK_a 's of key residues controlling the binding interaction. This technique should prove very useful in probing binding site structure on nonfunctioning targets such as enzyme zymogens as well as complicated receptor interactions.

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CHAPTER III

The Reaction of 3-Diazonium-1,2,4-triazole with Tryptophan
at Acid pH. A Scintillation Method for Tryptophan
Determination in Proteins.

ABSTRACT

Unlike conventional aryl diazonium reagents which are usually reactive at alkaline pH, 3-diazonium-1,2,4-triazole (3-DT) has been found to react with a variety of proteins at acid pH. Using indole, phenol and imidazole as models of aromatic amino acid side chains, the rate of 3-DT coupling to the indole nucleus has been shown to increase exponentially from $< 200 \text{ M}^{-1} \text{ min}^{-1}$ at pH 6 to $> 2400 \text{ M}^{-1} \text{ min}^{-1}$ at pH 2.5. Coupling rates with phenol and imidazole show the conventional alkaline reactivity, with no evidence of reaction at acid pH. In parallel experiments using trypsin, 3-DT was found to cause loss in enzymatic activity at acidic as well as alkaline pH. Furthermore, amino acid analysis has demonstrated that the acidic reaction with trypsin resulted in the modification only of tryptophan.

Based on the acidic specificity of 3-DT seen in these studies, a scintillation method for determining the tryptophan content of proteins has been developed. Using 3-diazonium-1,2,4-triazole-5- ^{14}C , accurate tryptophan contents for trypsin, chymotrypsin, lysozyme, pepsin, insulin and fibrinogen have been measured.

INTRODUCTION

Historically, diazonium reagents have been important tools in the development of protein chemistry. Most work has focused on the alkaline diazo coupling reactions of diazonium compounds first observed by Pauly in 1904 with diazotized p-sulfanilic acid. Pauly's discovery that diazo coupling reactions imparted coloration to histidine and tyrosine residues in proteins prompted a number of chemical studies which have elucidated the alkaline reactions of diazonium reagents with amino acids and proteins. These studies have been summarized by Horinishi et al. (1964). Briefly, histidine and tyrosine are capable of reacting with one or two equivalents of diazonium reagent at alkaline pH affording mono- and bis-azo derivatives. The bis-azo derivatives have absorption bands in the visible region, thus spectrophotometric assays for histidine and tyrosine content have been developed by quantitation of the bis-azo derivative formation (Tabachnick & Sobotka, 1959, 1960). The intense color of diazo-benzene sulfonic acid derivatives has also been used to locate and identify proteins and peptides following chromatography and electrophoresis procedures (Katsoyannis et al., 1967; Tometsko & Delihias, 1967). Diazo-1-H-tetrazole has been used to examine the differential reactivity of histidine and tyrosine residues in insulin (Horinishi et al., 1964; Takenaka et al., 1969). Unfortunately, in addition to coupling to tyrosine and histidine, diazonium reagents can also couple at neutral to alkaline pH with lysine, arginine, tryptophan, and the amino terminus of proteins yielding azo products with ultraviolet absorptions (Howard & Wild, 1957). The occurrence of multiple reaction sites has precluded the use of diazonium reagents in

the development of more sensitive amino acid assays and their use as specific alkylation reagents.

Since most diazonium reagents exhibit little or no reactivity below pH 6, literature dealing with their use at acidic pH is sparse. Recent work in this laboratory has exploited the known photosensitivity of diazonium compounds (Horner & Stohr, 1952) in a demonstration that diazo-benzene sulfonic acid may be used as a photoaffinity labeling reagent at acidic pH, where its diazo coupling reactivity is suppressed (Tometsko et al., 1978). Continuation of this work has led to the discovery that 3-diazonium-1,2,4-triazole (3-DT)¹, a reagent previously used only at alkaline pH, reacts specifically with tryptophan at acidic pH.

Previous workers have reported that 3-DT couples with proteins of chloroplast membranes at pH 7.7-8.9 yielding a change in turbidity and photochemical activity in chloroplast suspensions (Nakaya et al., 1970, 1972, 1974; Maruyama et al., 1974). The studies reported herein demonstrate that in addition to its expected alkaline reactivity, 3-DT covalently couples specifically to tryptophan below pH 6. Acidic reaction with 3-DT results both in functional inactivation of target proteins and in a loss of tryptophan recoverable by amino acid analysis. This is the first report of an indolyl-specific reaction of a diazonium compound at acid pH.

Based on the specificity of 3-DT for tryptophan, a scintillation method for the determination of the tryptophan content of nanomole quantities of purified proteins has been developed.

¹Abbreviations used: 3-DT, 3-diazonium-1,2,4-triazole; TCA, trichloroacetic acid; BNPS-skatole, 2-(2-nitrophenylsulfenyl)-3-methyl-3'-bromoindoleine.

EXPERIMENTAL PROCEDURES

Materials

3-Amino-1,2,4-triazole was obtained from Aldrich and recrystallized twice from ethanol (Sjostedt & Gringas, 1946) before use. 3-Amino-1,2,4-triazole-5-¹⁴C (6.1 mCi/mmol) was obtained from ICN Pharmaceuticals and stored refrigerated in water solution. Sodium nitrite (NaNO₂) was C.P. Baker "Analyzed" grade. Phenol, imidazole and indole were obtained from Aldrich and used without further purification. Glycyl-L-tryptophan and L-tryptophyl glycine were obtained from Sigma. Trypsin (twice crystallized, salt free, lyophilized), alpha chymotrypsin (3X), insulin and fibrinogen (65% clottable), all of bovine origin, were obtained from ICN Pharmaceuticals, Nutritional Biochemicals, Lilly Co., and Pentex Co., respectively. Hen egg lysozyme and porcine pepsin were obtained from Worthington and Schwartz Mann, respectively.

Buffer containing 0.1 M sodium succinate and 0.1 M sodium citrate was used in experiments where it was necessary to obtain stable values in the range of pH 2.5 to 8.5. Final adjustments to desired pH values were made with 1 N NaOH or 1 N HCl as required. Sample buffer for amino acid analyses was 0.20 N sodium citrate, pH 2.2, containing 0.5% thiodiglycol, 0.2% Brij-35 (polyoxyethylene lauryl ether), and 0.01% pentachlorophenol. No evidence for reaction of 3-DT with any component of the sample buffer has been observed.

Preparation of 3-DT

Stock reagent was prepared immediately prior to each experiment by reaction of 100 mg (1.19 mmol) of 3-amino-1,2,4-triazole with 82 mg

(1.16 mmol) of NaNO_2 in 5 ml of 1 N HCl. Diazotiazation proceeded for 30 minutes at 0° . A slight excess of aminotriazole has been routinely used to eliminate the need to destroy excess nitrous acid in the diazotization reaction. For studies of the pH dependence of 3-DT reactions, excess acid in the 3-DT stock was neutralized by titration to pH 3.5 with 6 N NaOH. 3-DT concentrations were calculated assuming complete diazotization.

3-DT is quite unstable and may present an explosion hazard in dry form (Polya, 1965; Melendez & Vilarrasa, 1974). Additionally, the compound is very sensitive to photolytic decomposition at near-UV wavelengths. All syntheses and subsequent handling of 3-DT should thus be performed in subdued room light.

UV Spectrum and Photosensitivity of 3-DT

Solutions of 0.30 mM 3-DT were prepared by dilution of stock 3-DT into 1 mM HCl or pH 6 buffer. Ultraviolet spectra were obtained for solutions which were unphotolysed or subjected to photolysis for various lengths of time. Photolysis was carried out by exposure to a Xenon flash lamp (filtered to cutoff wavelengths below 295 nm) as previously described (DeTraglia et al., 1978). All spectra were recorded on a Cary 219 spectrophotometer.

Treatment of Indole with 3-DT

A solution containing 0.4 mM 3-DT and 0.2 mM indole in pH 7 buffer was prepared by appropriate dilutions from 3-DT stock (previously neutralized to pH 3.5) and indole stock (5.0 mM in 5% ethanol, 95% water). The

UV spectrum of this solution was obtained immediately (500 to 250 nm at 1 nm/sec scan rate), then scanned repetitively for various times up to 40 minutes.

pH Dependence of 3-DT Reaction with Indole, Phenol and Imidazole

Solutions containing 5.00 mM indole, phenol or imidazole were prepared by dilution of 0.100 M stock solutions (in 5% ethanol, 95% water) into appropriate buffers between pH 2.5 and 8.5. Reaction was initiated by addition of 3-DT (previously neutralized to pH 3.5) to a final concentration of 0.3 mM. The rate of each reaction at 22° was measured by following the time course of change in absorbance at 370 nm in a Beckman DU spectrophotometer equipped with a chart recorder. Pseudo first order rate constants were determined by fitting the data to a linear exponential equation by the method of least squares (correlation constants were typically above 0.996). These rates were subsequently converted to bimolecular rate constants using the known concentrations of indole, phenol or imidazole, each in excess and assumed constant. The exact pH of each reaction mixture was measured after the kinetic data had been collected. Control experiments, in which 3-DT was mixed with an appropriate blank stock (5% ethanol, 95% water without other reagents) at each pH, failed to show evidence of reaction at 370 nm.

Treatment of Tryptophan with 3-DT

The stoichiometry of the reaction of 3-DT with free tryptophan at pH 2.2 was measured using a Beckman 120C amino acid analyzer with a ninhydrin detection system. Solutions containing 1.2 mM tryptophan and

varying concentrations of 3-DT were allowed to react for one hour at room temperature in pH 2.2 citrate sample buffer. Subsequently, 0.10 ml aliquots of these solutions were subjected to amino acid analysis on a 9 x 110 mm Aminex Q-15S column (Bio-Rad) developed at pH 5.28, 57°.

Inactivation of Trypsin with 3-DT

Trypsin solutions (0.15 mg/ml) were incubated at room temperature with 10 mM 3-DT in buffers ranging in pH between 2.8 and 6.8. At specified times, 50 μ l aliquots were withdrawn and assayed spectrophotometrically for tryptic activity by the hydrolysis of N- α -carbobenzoxy-L-lysine-p-nitrophenyl ester. All assays were performed at 22°, pH 6.2, monitoring nitrophenol formation at 322 nm, as previously described (DeTraglia et al., 1978). Tryptic activity was expressed as percent of control activity in the absence of 3-DT. Solutions were found to retain 100% activity when incubated at the test pH for specified times without 3-DT.

Amino Acid Recovery After 3-DT Reaction

Aliquots (5 ml) of a 5 mg/ml trypsin stock solution were mixed with 3-DT or 3-amino-1,2,4-triazole (control) at final concentrations of 10 mM in 0.1 N HCl. After 30 minutes at room temperature, four 1 ml aliquots were withdrawn from each of these solutions and transferred to small polypropylene tubes. Protein was precipitated by adding 0.25 ml of 25% TCA and holding the tubes on ice for 10 minutes. Samples were then centrifuged at 7000 xg for 10 minutes at 4°, the supernatant fluid was discarded, then the pellets were washed with 1 ml of 5% TCA and recentrifuged.

Two of the four pellets resulting from each reaction (3-DT or control) were subjected to complete alkaline hydrolysis and two to acid

hydrolysis as follows. Washed pellets were redissolved by the addition of 1 ml of 6 N HCl (acid hydrolysis) or 1 ml of 2 N KOH (alkaline hydrolysis). All polypropylene tubes were then placed into larger pyrex tubes, degassed under vacuum, purged with nitrogen, and sealed with teflon-lined screw cap closures. Hydrolysis was allowed to proceed for 22 hours at 110°. Alkaline hydrolysates were then acidified by addition of 3 ml of 0.8 N citric acid and 0.5 ml aliquots were analyzed for basic amino acids immediately on a Beckman 120C amino acid analyzer. Acid hydrolysates were evaporated to dryness under vacuum, redissolved in 4.0 ml of pH 2.2 citrate sample buffer, and 0.5 ml aliquots were analyzed for complete amino acid content. Amino acids recovered from the hydrolysates were identified and quantitated by comparison with a standard amino acid analysis profile (0.125 μ mol Pierce standard H + 0.125 μ mol Trp in pH 2.2 citrate sample buffer). Tryptophan content was determined from alkaline hydrolysates. All other amino acids were recovered in acid hydrolyses.

Treatment of Glycyltryptophan and Tryptophylglycine with 3-DT

To determine whether peptide bond cleavage at either side of tryptophan might occur concomitant with reaction of 3-DT at the tryptophyl indole nucleus, the reaction of 3-DT with two model dipeptides was studied. Solutions containing 2 μ mol/ml of 3-DT or 3-amino-1,2,4-triazole and 1 μ mol/ml of glycine, glycyl-L-tryptophan or L-tryptophylglycine were prepared in pH 2.2 citrate sample buffer and allowed to react at room temperature for 1 hour. Aliquots (0.2 ml) of these solutions were analyzed for free glycine content using an Aminex Q-155 column, as described above.

Determination of Tryptophan Content of Proteins with 3-DT

The following procedure has been used to determine the number of equivalents of 3-DT incorporated into several proteins at acidic pH. Based upon the specificity of 3-DT determined in the above experiments, 3-DT incorporation has been found to accurately measure the number of tryptophan residues in the test proteins.

1) Radiolabelling reagents were prepared by adding 2.5 μCi of 3-amino-1,2,4-triazole-5- ^{14}C to each of two tubes containing 1.0 ml of 0.100 M unlabeled 3-amino-1,2,4-triazole in 1 N HCl. One solution was used as a labeled aminotriazole control reagent, the other was converted to labeled 3-DT by reaction with 6.80 mg of NaNO_2 at 0° for 30 minutes. Cold 1 N HCl was used as a blank reagent.

2) Three 2.0 ml aliquots of protein stock solutions (0.5 mg/ml in 1 mM HCl) were mixed with 100 μl aliquots of blank reagent (1 N HCl), radiolabeled aminotriazole, or radiolabeled 3-DT. Two 200 μl aliquots of each of these solutions were withdrawn, one for protein assay and one for scintillation spectrometry (to determine the specific activity of labeled reagents). The remaining solutions were allowed to react for 30 minutes at room temperature.

3) After reaction, three 400 μl aliquots of each solution were subjected to protein precipitation by adding 100 μl of 25% TCA, vortexing, holding on ice for 10 minutes and centrifuging at 7000 $\times g$ for 10 minutes. Supernatant fluids were discarded and the procedure was repeated three more times using 500 μl of 5% TCA for washes.

4) Washed protein pellets were redissolved in 500 μ l of 0.25 NaOH. Two 200 μ l aliquots were withdrawn from each solution for protein assay and scintillation spectrometry.

5) Protein determinations were compared with standard curves constructed for each protein. Micrograms of protein recovered were converted to nanomoles based on the protein molecular weights. Scintillation counts were converted to nanomoles of label incorporated based on the specific activity of the labeled reagents (determined in Step 2). The ratio of nanomoles of 3-DT to nanomoles of protein in the pellet has been found to correspond to the number of tryptophan residues in the protein.

Scintillation cocktails contained 9.33 ml of scintillation fluid (prepared as 4 gm Omnifluor per liter of toluene) and 0.67 ml of Beckman BBS-3 solubilizer. Samples were counted at > 85% efficiency on a Beckman LS235 liquid scintillation counter using a wide ^{14}C window. Typically, background counts averaged 30 cpm, labeled aminotriazole washed out to 30-40 cpm, and incorporated 3-DT counted at 50-60 cpm per equivalent per nanomole of protein assayed. Protein determinations were done by the method of Toennies and Feng (1965), a modified Lowry procedure. A Bausch and Lomb Spectronic 710 spectrophotometer equipped with an automatic sample aspiration system was used to safely handle radioactive samples.

RESULTS

Spectra of 3-DT

An examination of the absorption spectrum of 3-DT was made: 1) to confirm that diazotization had occurred, 2) to determine the protonation state of the molecule at various acid strengths, and 3) to assess the rate of photolytic breakdown of the molecule. The UV spectrum of 3-DT after 0, 1, 3, 8 and 11 or more minutes of photolysis is shown in Figure 1. The compound absorbs maximally at 282 nm ($\epsilon_{282} = 2000 \text{ M}^{-1} \text{ cm}^{-1}$). First order photolytic breakdown of 3-DT occurs at a rate of $4.0 \pm 0.3 \times 10^{-4} \text{ pulse}^{-1}$ under exposure to a Xenon source operating at 14 pulses per second. The spectrum and photolytic breakdown rate are invariant over the range of pH 3 to 6.

Reaction of 3-DT with Indole

When 3-DT and indole are combined at neutral to acidic pH, a reaction takes place which results in strong yellow to red coloration. At pH 7, the reaction is sufficiently slow that its time course can be monitored by repetitive UV scanning, as shown in Figure 2. As the reaction proceeds, smooth conversion of 3-DT and indole to a product adduct is observed, with an isobestic point present at 311.5 nm. At more acidic pH, the reaction rate increases. This reactivity is a unique property of 3-DT, unobserved with other diazonium compounds (such as diazobenzene sulfonic acid).

pH Dependence of 3-DT Reactions

The pH dependence of the reaction rate for coupling of 3-DT with indole, phenol and imidazole has been determined. These compounds were

Figure 1: UV spectrum of 0.30 mM 3-DT obtained at pH 3. Spectral curves represent the result of 0, 1, 3, 8 and 11 or more minutes of photolysis. Photolytic breakdown of 3-DT may be characterized as a first order process as evidenced by linearity of the logarithmic plot of 3-DT absorbance versus photolysis time.

Figure 2: UV spectra for the time course of formation of a reaction adduct between 0.40 mM 3-DT and 0.20 mM indole at pH 7. After mixing reagents, repetitive scans were initiated at the times indicated (min). The broken line represents the spectrum of 0.40 mM 3-DT in the absence of indole.

chosen in order to evaluate the expected reactivity of 3-DT with tryptophan, tyrosine and histidine side chains without the complications of free amine reactivity which might occur at alkaline pH. Reaction rate was determined by monitoring the increase in absorbance at 370 nm which occurs during the coupling. This wavelength is at the maximum absorbance of the 3-DT/indole adduct, and near the 360 nm maximum for the monoazo derivatives of 3-DT with tyrosine and histidine (Nakaya et al., 1970). As shown in Figure 3, 3-DT shows the expected reactivity of diazonium compounds toward phenol (tyrosine) and imidazole (histidine), with little reactivity below pH 6. In striking contrast, the coupling rate with indole increases exponentially below pH 6 with little reactivity at alkaline pH. This behavior imparts a great deal of selectivity to 3-DT at acidic pH, where only the indole nucleus is an active coupling target.

Reaction of 3-DT with Tryptophan

The selectivity toward indole coupling at acidic pH suggested that 3-DT might be useful as a specific alkylating agent for tryptophan in protein. The stoichiometry of the 3-DT coupling reaction with tryptophan was studied by amino acid analysis. Figure 4 presents elution tracings from amino acid analyses which indicate that 3-DT reacts one to one with tryptophan. Addition of 3-DT causes a stoichiometric decrease in the tryptophan band occurring at 28 minutes in the elution profile, and a corresponding increase in a rapidly eluting band with very low ninhydrin color yield at 13 minutes. Horinishi et al. (1964) have reported that tryptophan reacts with no more than one equivalent of diazonium compounds at neutral pH.

Figure 3: pH dependence of coupling rates between 3-DT and indole ($\square$), phenol ($\bigcirc$) and imidazole ($\triangle$) determined under pseudo first order conditions.

Figure 4: Elution profiles from amino acid analyses of tryptophan solutions following reaction with varying amounts of 3-DT at pH 2.2 for 1 hour. In all cases, 100 μ l of test solution was applied to the analyzer. Traces A, B and C represent solutions containing 1.2 mM Trp with 0.0, 0.2 and 1.0 mM 3-DT respectively. Integrations of the Trp elution bands (at 28 min.) yielded recoveries of 0.12, 0.10 and 0.04 μ mol of tryptophan respectively. Solid (570 nm) and broken (440 nm) lines represent ninhydrin absorbance on a logarithmic ordinate.

Treatment of Trypsin with 3-DT

When trypsin solutions were incubated with 3-DT, coloration of the protein occurred, and a loss of enzymatic activity with time was observed at all pH values studied (pH 2.8 - 6.8). As shown in Figure 5, activity is lost faster at pH 3.6 than at pH 4.9. The minimal rate of inactivation was at pH 5.2, consistent with the minimal reactivity of 3-DT at this pH seen in studies of coupling rates (Figure 3). As shown in Figure 6, the tryptic activity decays exponentially, with inactivation proceeding more rapidly below pH 5.2. Inactivation conforms to a first order decay process, evidenced by a linear logarithmic plot. At more alkaline pH (above pH 5.2), the rate of loss of tryptic activity again increases substantially, but fails to conform to a single exponential decay equation, presumably due to the mixed action of 3-DT on tyrosine, histidine and free amino groups.

The recovery of amino acids from trypsin after reaction with 10 mM 3-DT in 0.1 N HCl (pH 1) was determined by analysis of complete acid and alkaline hydrolysates. All amino acids except tryptophan were recovered quantitatively (Table I). Loss in tryptophan was accompanied by the appearance of an early elution band occurring at 13 minutes in the acid hydrolysate, representing the 3-DT/Trp adduct (see Figure 4).

Reaction of 3-DT with Model Dipeptides

A number of mild oxidizing agents, such as N-chlorosuccinimide (Shechter et al., 1976), and BNPS-skatole (Omenn et al., 1970; Martenson et al., 1977) are known to react with the tryptophyl indole nucleus and produce concomitant cleavage of tryptophyl peptide bonds. To determine whether 3-DT reaction might also result in peptide bond cleavage, the

Figure 5: Time course of activity loss of trypsin solutions incubated with 30 mM 3-DT at pH 4.9 (●) or pH 3.6 (○). Trypsin solutions (0.16 mg/ml) were incubated with 30 mM 3-DT in 0.05 M succinate buffers of specified pH. Aliquots were withdrawn and assayed at specified times by measuring the rate of tryptic hydrolysis of N- α -carbobenzoxy-L-lysine-p-nitrophenyl ester at pH 6.2.

Figure 6: pH dependence of the inactivation of trypsin by 3-DT. Reaction mixtures contained 0.15 mg/ml trypsin with 10 mM 3-DT and were incubated at room temperature at the specified pH. Assays were performed as in Figure 5.

Table I
RECOVERY OF AMINO ACIDS FROM TRYPSIN AFTER ACIDIC REACTION WITH 3-DT^a

Amino Acid	Control (μ mol)	3-DT (μ mol)	Percent Recovery
Tryptophan ^b	0.09	< 0.01 ^c	< 11
Lysine	0.24	0.25	104
Histidine	0.06	0.06	100
Ammonia	0.55	0.63	115
Arginine	0.04	0.04	100
Aspartic Acid + Asparagine	0.41	0.40	98
Threonine	0.19	0.18	95
Serine	0.57	0.54	95
Glutamic Acid + Glutamine	0.26	0.26	100
Proline	0.15	0.15	100
Glycine	0.47	0.47	100
Alanine	0.27	0.26	96
Valine	0.27	0.25	93
1/2 Cystine ^d	-	-	-
Methionine	0.03	0.03	100
Isoleucine	0.24	0.23	96
Leucine	0.25	0.25	100
Tyrosine	0.15	0.14	93
Phenylalanine	0.06	0.06	100

^a Experimental details are presented in the text.

^b Trp content from alkaline hydrolysates was normalized to Lys content in same sample and the corresponding acid hydrolysate.

^c In addition to the loss of tryptophan observed in KOH hydrolysates, 3-DT reaction produced a small elution band at 13 min. (observable only in the HCl hydrolysate). Presumably, this is the same 3-DT/Trp adduct observed in Figure 4.

^d Cysteine and cystine were not recovered by this hydrolysis procedure.

products of the reaction of 3-DT with glycyl-L-tryptophan and L-tryptophylglycine were analyzed. No free glycine was detected by amino acid analysis after reaction of the dipeptide solutions with 3-DT at pH 2.2. In control experiments it was found that 3-DT had no effect on analysis of glycine standards. Thus it does not appear that the reaction of 3-DT with tryptophan is accompanied by cleavage of adjacent peptide bonds.

Incorporation of 3-DT into Proteins

The number of equivalents of radiolabeled 3-DT incorporated into several proteins of known sequence has been determined by the scintillation method outlined. As seen in Table II, the incorporation of 3-DT at acid pH appears to be an accurate determinant of tryptophan content. Proteins used in the assay ranged in molecular weight from 6000 to 340,000 with known tryptophan contents from 0 to 78 per molecule. No serious difficulties were encountered with any of the proteins, although pepsin required a higher TCA concentration (20%) to prevent losses in recovery from the precipitation and wash protocol. Furthermore, 3-DT appears to be extremely reactive and selective, requiring only mild conditions (dilute acid, room temperature, 30 minutes) to achieve complete coupling with tryptophan in proteins.

Table II

DETERMINATION OF TRYPTOPHAN CONTENT IN PROTEINS BY INCORPORATION
OF RADIOLABELED 3-DT

Protein	Molecular Weight	3-DT/protein found	Trp theor	Reference
Insulin	6,000	0.1 ± 0.1	0	
Lysozyme	14,400	6.0 ± 0.1	6	Canfield & Liu (1965)
Trypsin	23,800	4.2 ± 0.2	4	Mikes et al. (1966)
Chymotrypsin	25,000	8.2 ± 0.1	8	Hartley & Kaufman (1966)
Pepsin ^a	35,000	4.6 ± 0.4	5	Sepulveda et al. (1975)
Fibrinogen ^b (65% clottable)	340,000	51.9 ± 2.1 (67%)	78	Ishida et al. (1978)

^aPepsin required 20% TCA for precipitation and washes.

^bFibrinogen used in this experiment was reported to be >65% protein by weight, and 65% clottable. The number of equivalents of 3-DT incorporated represents 67% of the theoretical number for 100% protein.

DISCUSSION

The use of a novel aryl diazonium compound, 3-diazonium-1,2,4-triazole (3-DT), as a selective reagent for the modification of tryptophan at acid pH has been investigated. Above pH 6 the reactivity of 3-DT parallels that of conventional aryl diazonium reagents; a finding corroborated 1) by kinetic studies of coupling to phenol and imidazole (Figure 3), 2) by studies of the inactivation of trypsin above pH 6, and 3) by previous studies in the literature using 3-DT (Nakaya et al., 1970, 1972, 1974; Maruyama et al., 1974). The present study demonstrates that unlike conventional aryldiazonium reagents, 3-DT is extremely reactive with indolyl moieties at acid concentrations below pH 5. Furthermore, the selectivity of 3-DT at acid pH has been demonstrated by its failure to react with amino acids other than tryptophan in trypsin. This reactivity and selectivity together with the one to one stoichiometry of the 3-DT reaction with tryptophan, has enabled measurements of the tryptophan content of several proteins to be made based upon incorporation of radiolabeled 3-DT.

Although the tryptophan content of many proteins is very small, the importance of tryptophan in stabilizing protein conformation via hydrophobic interactions is well established. Methods to study the functional consequences of selective tryptophan modification are thus of recognized importance in protein chemistry. Unfortunately, the availability of tryptophan specific reagents is limited. BNPS-skatole, developed as a selective tryptophan oxidant and cleavage reagent for tryptophyl carboxyl peptide bonds (Omenn et al., 1970) has recently been found also to react with tyrosine, the tryptophyl amino peptide bond, and to yield a mixture of indole oxidation

products (Martenson et al., 1977). N-chlorosuccinimide, though specific in its cleavage of tryptophyl peptide bonds, oxidizes methionine and cysteine as well (Shechter et al., 1976). Studies presented herein demonstrate that the acidic reaction of 3-DT with trypsin results only in tryptophan modification. Furthermore, reaction of 3-DT with tryptophyl dipeptides has not resulted in tryptophyl peptide cleavage.

Assessing the number of tryptophan residues present in a protein is often very difficult. Acid hydrolysis destroys tryptophan, and the variabilities encountered in alkaline hydrolysis often yield inaccurate determinations unless tedious precautions are taken (Hugli & Moore, 1972). Spectrophotometric methods involving sulfenyl halides (Scoffone et al., 1968) or 2-hydroxy-5-nitrobenzyl bromide (Barman & Koshland, 1966) have been most successful. Unfortunately, these methods must rely on spectrophotometric selection of the tryptophyl adducts, as other amino acids are modified which do not attain coloration. Scintillation measurements using these reagents are not possible. Thus the use of radiolabeled 3-DT for tryptophan determinations may provide a significant enhancement in the sensitivity of determinations on small samples of protein. Indeed, whereas micromole quantities of protein are required for spectrophotometry, several nanomoles are sufficient for the scintillation method outlined. Single determinations contributing to Table II have been conducted on 200 μ g samples, representing 0.5 (fibrinogen) to 33 (insulin) nanomoles of protein. Additionally, 3-DT seems to find access to interior hydrophobic regions of protein readily. This has eliminated the need for pretreatment to denature proteins before assay.

The detailed mechanism for the reaction of 3-DT with indolyl compounds at acid pH has not yet been determined. Much of the peculiar reactivity of the reagent can probably be accounted to its unusually low pK_a of 0.3 for diazo $\rightleftharpoons$ diazonium equilibrium (Melendez & Vilarrrasa, 1974). Results in the present study confirm that the UV spectrum of 3-DT is unchanged from pH 3 to 6, consistent with a pK_a outside of this range. Furthermore, the neutral charge character of 3-DT in this pH range may account for the ease with which it penetrates to the interior hydrophobic regions of protein. Attempts to purify 3-DT as a diazonium tetrafluoroborate salt have been unsuccessful, again presumably due to the neutral charge character of the compound.

Coupling reactions of amines and imidazolyl compounds are known to occur via interactions with their un-ionized forms, and coupling of phenolic residues is believed to involve the phenolate anion (Zollinger, 1961). Thus it is not surprising that these residues are unreactive at acidic pH. Coupling with indole, however, involves reaction with the neutral indole molecule without previous formation of its conjugate base (Binks & Ridd, 1957). Thus indole would be expected to couple at acidic pH, provided the diazonium reagent maintained acidic reactivity. Diazo coupling to indole may directly result in colored C-azo products (Binks & Ridd, 1957), or may result from acidic rearrangement of triazenes formed by reaction at the amino nitrogen (Howard & Wild, 1957; Zollinger, 1961). Studies with a number of indole derivatives are presently being undertaken to elucidate the mechanism of the acidic 3-DT reaction, and to ascertain the exact structure of the reaction products.

Plate 1: Diazo-diazonium equilibrium of 3-diazonium-1,2,4-triazole.

In summary, this work demonstrates that 3-DT may be used as a specific reagent for tryptophan in analytical and modification studies at acidic pH. As this is the first report of such specificity using a diazonium reagent, further study of the mechanism of reaction of indolyl compounds with 3-DT is warranted.

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APPENDIX

The technique used in this thesis to compute equilibrium binding affinities from spectrophotometric titration data relies on an absolute method for the spectroscopic determination of equilibrium constants developed by Rose and Drago (J. Am. Chem. Soc. 81: 6138-6145, 1959). This method remains applicable when the concentrations of enzyme and ligand are nearly equal, a condition which excludes the use of Eadie or Scatchard analysis. The equations are general, and can be applied to any singly-liganded complex that absorbs or fluoresces in some region of the spectrum.

The general equation for the equilibria involved is

where E and L are enzyme and ligand, respectively. The equilibrium constant expression for this reaction is

$$K_d = \frac{[E][L]}{[EL]} = \frac{([E]_0 - [EL])([L]_0 - [EL])}{[EL]} \quad (2)$$

where [EL] = concentration of the complex at equilibrium; [E] = concentration of the free enzyme at equilibrium; $[E]_0$ = formal concentration of enzyme present in the reaction; [L] = concentration of the free ligand at equilibrium; and $[L]_0$ = formal concentration of ligand added to the reaction.

When the ligand molecule does not absorb in the region studied, or when difference spectra are utilized to subtract the ligand absorption, equation (3) shows the relationship of the total absorbance, A, to the free enzyme concentration and to the complex concentration, at any given wavelength:

$$A = \epsilon_c l [EL] + \epsilon_E l [E] \quad (3)$$

where ϵ_c = molar absorptivity of the complex; ϵ_E = molar absorptivity of the enzyme; and l = cell path length in cm. When only one complex is formed, equation (4) is also characteristic of the system.

$$[E]_0 = [EL] + [E] \quad (4)$$

If equation (2) is expanded

$$K_d = \frac{[E]_0 [L]_0 - [EL] [L]_0 - [E]_0 [EL] - [EL]^2}{[EL]} \quad (5)$$

Eliminating $[E]$ between equations (3) and (4) allows the absorbance to be expressed as

$$A = [EL]l(\epsilon_c - \epsilon_E) + [E]_0 l \epsilon_E \quad (6)$$

If A_0 corresponds to the absorbance of the initial concentration of enzyme, then according to Beer's law, $A_0 = \epsilon_E l [E]_0$ and then

$$A = [EL]l(\epsilon_c - \epsilon_E) + A_0 \quad (7)$$

Equation (7) is solved for $[EL]$ then $[EL]$ is eliminated between this solution and equation (5). After simplification,

$$K_d = \frac{[E]_0 [L]_0 (\epsilon_c - \epsilon_E) l}{A - A_0} - [E]_0 - [L]_0 + \frac{A - A_0}{(\epsilon_c - \epsilon_E) l} \quad (8)$$

or

$$K_d = \frac{[E]_0 [L]_0 l \Delta \epsilon}{\Delta A} - ([E]_0 + [L]_0) + \frac{\Delta A}{l \Delta \epsilon} \quad (9)$$

where the two unknowns K_d (the equilibrium constant) and $\Delta \epsilon$ (the change in molar absorptivity on going from free E to EL complex) are related to the experimental observable ΔA (the difference in absorbance, A , of the solution when only free enzyme is present, $[L]_0 = 0$, and when the formal concentration of ligand is non-zero), l (the cell path length), and the formal concentrations $[E]_0$ and $[L]_0$.

It should be noted that when $[L]_0 \gg [E]_0$ then $[L] \approx [L]_0$ and $[EL] - [E]_0$ is negligible when compared with $\frac{[E]_0 [L]}{[EL]} - [L]$. Imposing this condition and noting that $[EL] = \frac{\Delta A}{l \Delta \epsilon}$, equation (9) reduces to

$$K_d = \frac{[E]_0 [L]}{[EL]} - [L] \quad (10)$$

or

$$[EL] = [E]_0 - K_d ([EL]/[L]) \quad (11)$$

Eadie and Schatchard analyses rely on variations of equation (11); recalling the condition $[L]_0 \gg [E]_0$.

Returning to the general case resulting in equation (9): as the solution containing E is titrated with L, the change in absorbance from the initial value (ΔA) is observed. Thus a set of simultaneous equations in the two unknowns K_d and $\Delta \epsilon$ is generated. If the set contains more than two equations (as is the case for a multiple-point titration), K_d and $\Delta \epsilon$ are over determined. The solution is thus achieved by an iterative least squares approach. An initial value of K_d is assumed, from which a "best" value of $\Delta \epsilon$ can be calculated by the equation

$$\Delta \epsilon_{\text{best},j} = \frac{\sum_{i=1}^n (\Delta A_i / l) [EL]_{ij}}{\sum_{i=1}^n [EL]_{ij}^2} \quad (12)$$

where n is the number of points in the titration and $[EL]_i$ is the concentration of the complex present when the formal concentrations of E and L are $[E]_0^i$ and $[L]_0^i$ and is calculated from the expression

$$[EL]_{ij} = - ([E]_0^i + [L]_0^i + K_{d,j}) + \sqrt{([E]_0^i + [L]_0^i + K_{d,j})^2 - 4 [E]_0^i [L]_0^i} \quad (13)$$

Values of $\Delta A_{i,j,calc}$ are then calculated from $\Delta \epsilon_{best,j}$ using the relationship $\Delta A_{i,j,calc} = [EL]_{ij} \Delta \epsilon_{best,j}^{-1}$. The least squares sum,

$$\sum_{i=1}^n (\Delta A_{i,j,calc} - \Delta A_i)^2 = DEV_j$$

is then calculated and compared with DEV_{j-1} .

If $DEV_j < DEV_{j-1}$, the refinement parameter R is doubled and added to

$K_{d,j}^{-1}$ to give a new estimate of K_d . The cycle is repeated until $DEV_j > DEV_{j-1}$

at which time the sign of R is reversed and its magnitude decreased by a

factor of two. This represents the initial portion of the iteration. From

this point onward, R is halved and its sign reversed each time $DEV_j - DEV_{j-1}$

changes sign. Iteration is halted when $R_j K_{d,j} \leq \delta$, an error limit set for

the determination of K_d .