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Aliphatic Diazirines as Photoaffinity Probes for Proteins: Recent Developments

Joydip Das*

Department of Pharmacological and Pharmaceutical Sciences, College of Pharmacy, University of Houston, Houston, Texas 77204, United States

CONTENTS

1. INTRODUCTION

Diazirines are three-membered ring systems containing two nitrogen atoms and one carbon atom[1,2] widely applicable in biology as photoaffinity labeling agents.[3,4] Diazirines can be categorized as two types: aliphatic and aromatic. In aliphatic diazirines, the cyclic ring system is directly attached to aliphatic carbons, whereas in aromatic diazirines, it is directly attached to an aromatic ring through the diazirine ring carbon atom. Although diazirines were first chemically synthesized in 1960[5,6] and its structure was confirmed in 1962,[7,8] the potential of diazirines as photoprobes was first revealed much later in the 1970s by Jeremy Knowles.[9−11] In 1980, Brunner et al.[12] modified an aromatic diazirine by introducing a trifluoromethyl group to the diazirine carbon and demonstrated its promise as an effective photolabeling agent. This discovery triggered synthesis of many biologically active ligands containing aromatic diazirine moiety broadening their use as photoprobes for proteins otherwise difficult to crystallize, for nucleic acids and organic polymers. However, the use of aliphatic diazirines remains limited.[13−18] The apprehension primarily rests on the following issue. Photolysis of aliphatic diazirines produces long-lived photointermediates that can diffuse from its site of generation complicating photolabeling data interpretation. However, there has been a steady increase in the use of aliphatic diazirines in the recent past. Even the simplest form of diazirine where the diazirine moiety is attached to two hydrogens (Figure 1) has been used to probe folding and unfolding states of proteins.[19−21] This paper reviews the mechanistic aspects of photolabeling, examples of aliphatic diazirines as biological photoprobes, and methods of detection of photolabeling and discusses important caveats in aliphatic diazirine research.

2. MECHANISM OF PHOTOLABELING: AROMATIC DIAZIRINES VERSUS ALIPHATIC DIAZIRINES

The characteristics of an ideal photoprobe are stability in the dark at various pH values, close resemblence to the parent molecule with similar affinity and less steric perturbation, activation at wavelengths that do minimal or no damage to the other components of the system and generation of photointermediates that are highly reactive (short-lived), and ability to react with any type of bond or residues without any preference and to form stable adducts with the receptor to survive detection methodology. All of these criteria are hard to meet in a single photolabeling agent. Therefore no agents are ideal. While aliphatic diazirines, due to their smaller size, are preferred, the photochemistry of aromatic diazirines makes these more popular. Generally, upon photoirradiation diazirines generate carbenes, which in most cases undergo insertion into amino acid residues resulting in photolabeling. Carbenes are highly reactive species with lifetimes in the range of nanoseconds.[22,23] In addition to generating carbenes, diazirines undergo isomerization generating linear diazo compounds (Figure 2). Diazo compounds have a much longer lifetime than carbenes, are less reactive and are sensitive to protonation, and can generate carbene or carbocation that can label the nucleophilic residues of protein. Because of its longer lifetime, diazo compounds can diffuse from an initial site of generation to other sites in the protein. The formation of the carbene and the diazo compounds are characterized both chemically[12,24,25] and spectroscopically.[26] The introduction of

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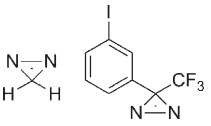

Figure 1. 3*H*-Diazirine and trifluoromethyliododiazirine (TID).

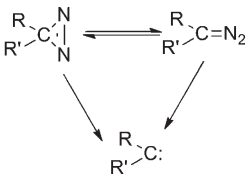

Figure 2. Oversimplified scheme for the photoreaction of diazirines. Carbene and diazo compounds are reactive species for the photoaffinity labeling of proteins.

a trifluoromethyl group to the diazirine ring carbon can stabilize the carbene preventing it from undergoing rearrangements. Moreover, due to the electron-withdrawing nature of this group, the photogenerated diazo isomer is so strongly stabilized that it can be considered to be an unreactive species under normal photolysis conditions. The diazo compounds generated from aliphatic diazirines are unstable and prone to generate carbocations that can react with the nucleophilic side chain containing amino acids, causing nonspecific photolabeling.

Recent studies using ultrafast spectroscopy indicate that the photochemistry of diazirines is complex. More photointermediates other than carbenes and diazo compounds may exist. The complexity of the photochemistry is attributed to the generation of other photoproducts such as alkenes formed by the rearrangement in the excited state (RIES) upon photoirradiation. Figure 3 shows a current scheme that includes the possible pathways and intermediates found in diazirine photochemistry. When diazirines are used as a source of carbenes, several processes contribute to the formation of the photoproduct, including one arising from the rearrangement of the excited state of diazirine, and a metastable diazo intermediate, which competes with the formation of carbenes. The relative yield of these intermediates, carbene, diazo, and the rearrangement products are dependent on the chemical nature of the substituent in the diazirine molecule. However, several studies indicate that aromatic diazirines tend to generate more carbenes compared with aliphatic diazirines.[12,24,27−29] For example, for 3-trifluoromethyl-3-phenyldiazirine, it has been estimated that about 65% of the diazirines are converted to the carbenes, while 35% were converted to 1-phenyl-2,2,2-trifluoromethyl-3-diazomethane, based on their UV−vis absorption spectra.[12] In contrast, photolysis of an aliphatic diazirine, 3,3,-bis(1,1-difluorohexyl)diazirine, produced 56% diazo compound, and 40% of the products were found to be derived from the carbenes.[24] In the case of adamantyl diazirine, the quantum yields for formation of the corresponding carbene and diazo compounds were both around 50%.[27,28] For isopropylchlorochlorodiazirine, carbene, diazo, and the RIES alkene products form 5%, 13% and 82% of the total product, respectively.[29] For chloromethylchlorodiazirine, carbene and alkene from RIES are formed in 36% and 64% yields, respectively.[29] In the case of phenylchlorodiazirine, the carbene yield is 99%.[28]

Photolysis of diazirines can generate two different types of carbenes, singlet carbene, where the two electrons with antiparallel spins are in the same orbital, and triplet carbene, where two electrons that have parallel spins are occupied in two different

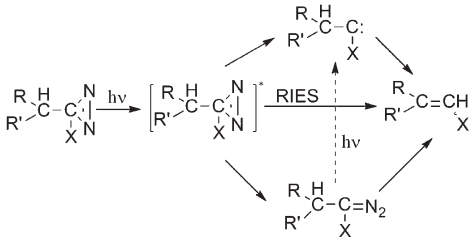

Figure 3. Pathways of diazirine photolysis. In addition to generating carbenes and diazo compounds, diazirines are converted to alkenes by a mechanism called rearrangements in the excited state (RIES). All these intermediates were characterized by chemical and spectroscopic methods.

orbitals. Typically, singlet carbenes react rapidly (bimolecular rate constants of 10^7-10^9 M^{-1} s^{-1}) with most functional groups, including alcohols, aromatics, alkenes, and even normally unreactive C−H bonds to form stable adducts. The triplet carbene cannot insert into an O−H bond, because that would involve the formation of an unstable −O• radical species, but can insert into the C−H bond. A carbene species that has a small energy gap between its singlet and triplet state can equilibrate rapidly and display indiscriminate insertion reactions. The ground state of the parent carbene (:CH2) is a triplet due to lower Coulombic repulsion between the nonbonding electrons as they occupy different orbitals with parallel spins. The energy difference between the lowest singlet and the triplet ground state of carbene is ∼8−10 kcal/mol.[30−33] Thus, in general, the triplet is the more stable state (the ground state) and singlet is the excited state species. Substituents that donate electron pairs may stabilize the singlet state by delocalizing the nonbonding pair of electrons into an empty *p*-orbital, and if the energy of the singlet state is sufficiently reduced, it may become the ground state. Methyl and phenyl substituents can also selectively stabilize the lowest singlet, reducing the energy difference to 2−4 kcal/mol, but the triplet remains the ground state of methylcarbene and phenylcarbene.[34] Nevertheless, most of the solution-phase chemistry of these carbenes derives from a low-lying, readily accessible, highly reactive singlet state. The photoproduct of several aromatic diazirines was analyzed, and it was concluded that the majority of the products originated from the singlet state.[25,35,36] In the photolysis of a diazirine analog of phenylalanine, generation of a trace amount of triplet carbene in alcoholic solution was predicted.[25]

In summary, the reactive species for diazirine photolabeling are primarily singlet carbene and the diazo compound, the proportion of which depends upon the chemical nature of the diazirine.

3. ALIPHATIC DIAZIRINES AS BIOLOGICAL PROBES

Aliphatic diazirine analogs of several biologically important ligands have been employed as ligand binding site probes and for the determination of the topology of membrane-bound proteins. The aliphatic diazirines can be categorized according to the type of parent ligand molecule; a representative chemical structure is shown for each category. While most of the earlier studies detected only photolabeled peptides, advancement of Edman and mass spectrometric sequencing methods permitted identification of labeled residues in most of the recent studies. All photolabeled residues detected with aliphatic diazirines are listed in Table 1.

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Table 1. Summary of the Protein Residues Detected Using Aliphatic Diazirines

protein	aliphatic diazirine	residues	refs
PKC deltaC1B	3-azioctanol	Tyr-236	13
	7-azioctanol	Tyr-236	
	7-azioctanol	Tyr-236	
PKC deltaC1A	3-azioctanol	Tyr-187, Glu-160, Lys-198	15
	7-azioctanol	Tyr-187, Glu-160, Lys-198	
	7-azibutanol	Tyr-187, Glu-160, Lys-198	
adenylate kinase	3-azibutanol	Tyr-32, Tyr-34, His-36	16
	3-azioctanol	His-36	
	7-azioctanol	Asp-41	
PKC epsilonC1B	3-azibutanol	His-248	14
	3-azioctanol	His-248, Tyr-250	
nicotinic acetylcholine receptor	azietomidate	Glu-262, Tyr-93, Tyr-190, Tyr-198 (α); Gln-276, Ser-258, Ser-262, Asp-59 (δ)	18
nicotinic acetylcholine receptor (time resolved photolabeling)	azietomidate	Glu-262, Glu-390, His-408, Cys-412(α); Asp-268, Glu-272 (β); Gln-276, Cys-236, Glu-280 (δ)	64
nicotinic acetylcholine receptor	3-azioctanol	Glu-262, His-408, Cys-412, Tyr-190, Tyr-189, Ala-403 (α)	46
$GABA_A$	azietomidate	Met-236 (α1), Met-286 (β3)	65
MUNC13.1 C1	3-azioctanol	Glu-582, Asp-615, Glu-599	48
	3-azibutanol	Glu-582, Tyr-581, Glu-584, Asp-615, Tyr-579	
L1	3-azioctanol	Tyr-418, Glu-33	44
	3-azibutanol	Tyr-418, Glu-33	
apoferritin	*H*-diaziflurane	Leu-24, Ser-27	50
horse spleen apoferritin	azi-isoflurane	Arg-59	52
integrin LFA-1	azi-isoflurane	Tyr-257	52
nicotinic acetylcholine receptor	azicholesterol	Glu-398, Asp-407, Cys-412 (α); Asp-436, Asp-457 (β); Asp-448 (γ); Asp-454 (δ)	84
Rho GTPase	3-azioctanol	Glu-163	45
	3-azibutanol	Glu-193, Glu-163	
transglutaminase	3-(3-methyl-3*H*-diazirin-3-yl)-*N*-{4-[(*E*)-3-oxo-3(pyridin- 3-yl)prop-1-enyl] phenyl}propanamide	Cys-230	17
β-hexosaminidase B	3-azi-1-[(2-acetamido-2-deoxy-1-β-D-galactopyr nosyl)thio]butane	Glu-355	63

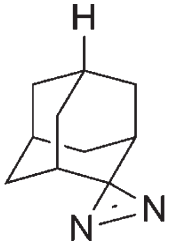

Figure 4. Adamantyldiazirine.

3.1. Diazirine Analogs of Adamantane

Adamantylidene is an effective lipophilic reagent used for the identification of hydrophobic, membrane-bound segments of intrinsic membrane proteins.[37] The diazirine analog generates a carbene interemediate that cannot undergo olefin formation at the bridgehead, which prevents carbene from undergoing rearrangement processes. This photolabel (Figure 4) was used for studying topology of Na/K ATPase,[38,39] Ca-ATPase,[40] and cytochrome P450 2B4[41] in membrane. The diazirine analog of a similar bridged compound, diamantine, was evaluated as a probe for P450 2B1.[42] However, it must be noted that in these studies labeled amino acid residue(s) were not detected.

3.2. Diazirine Analogs of Alcohols

Azialcohols (Figure 5) were used to study alcohol and anesthetic binding sites in soluble and membrane bound proteins. 3-Azibutanol and 3- and 7-azioctanols were used as model compounds for alcohol and anesthetics that interact with soluble proteins such as PKC subdomains,[13−15,43] L1,[44] RhoGDPase,[45] and membrane bound ligand-gated ion channels (LGICs) such as nicotinic acetylcholine[46,47] receptors. Diazirine analogs showed similar receptor activation or inhibition properties to those of the parent alcohols. While aziethanol, the diazirine analog of ethanol, would be the ideal molecule to study ethanol binding site in proteins, this analog had never been synthesized, most likely due to the inherent instability associated with this molecule. Azibutanol is the stable and the smallest azialcohol that serves as a surrogate for ethanol in the receptor studies.

Previously, in an elegant study, two photoactivable general anesthetics, *n*-octan-1-ol geometric isomers bearing a diazirine group on either the third or seventh carbon (3- and 7-azioctanol, respectively), were used to locate and delineate an anesthetic site

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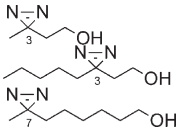

Figure 5. Azialcohols, 3-azibutanol (top), 3-azioctanol (middle), and 7-azioctanol (bottom).

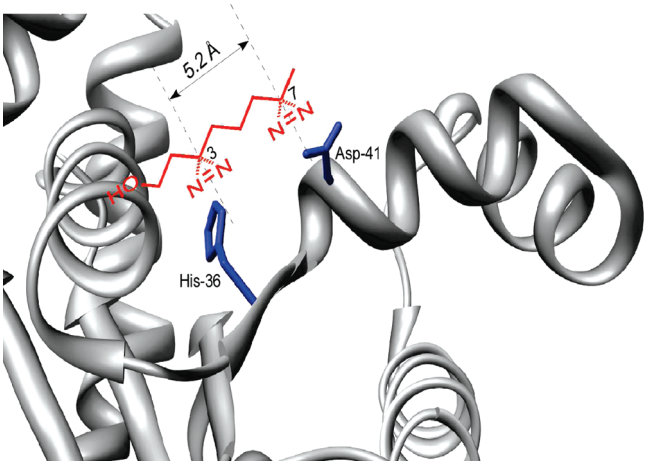

Figure 6. The octanol binding site on adenylate kinase. A portion of the protein structure showing the location of the photolabeled residues His-36 and Asp-41, which are photolabeled by 3- and 7-azioctanol, respectively. The closest approach of their side chain is ~5 Å, and the maximum distance between the 3rd and 7th carbon atoms is 5.2 Å. The azioctanol is modeled as a fully stretched molecule where diazirine group can be either at the 3rd or 7th carbon atom.

on adenylate kinase, a surrogate for excitable membrane proteins.[16] Both isomers were reported to be photoincorporated into the protein at 1:1 mol ratio. 3-Azioctanol photolabeled His-36, whereas 7-azioctanol photolabeled Asp-41.[16] Examination of the known structure of adenylate kinase revealed that the side chains of these residues are within ~5 Å of each other, a distance that matches the separation of 3- and 7-positions of an extended aliphatic chain (Figure 6). This indicated that azialcohols could serve both as microscopic ruler and environment-sensitive probe for proteins. The alkanol site spans two domains of adenylate kinase where His-36 is part of the CORE domain and Asp-41 belongs to the nucleotide monophosphate binding domain. In contrast, azibutanol was photoincorporated into several residues including His-36, which was also photolabeled by azioctanol.

In protein kinase C (PKC) and related proteins, however, the positional isomers 3- and 7-azioctanol were not able to delineate the residues in the alcohol binding site. Tyr-236,[13] Tyr 187,[15] and Glu-582[48] were preferentially labeled in PKCδC1B, PKCδC1A and Munc 13.1 C1, respectively, by all three azialcohols, 3-azibutanol and 3- and 7-azioctanol. In the case of PKCεC1B, however, relatively more hydrophilic azibutanol preferentially labeled His-248, which is somewhat surface exposed, and hydrophobic azioctanol preferentially labeled Tyr-250, which is buried inside the protein.[14]

Azialcohols are also used to probe interaction between alcohols and Rho GDP dissociation inhibitor (RhoGDIR), which is a mammalian signal transduction protein and binds to the geranylgeranyl moiety of GDP-bound Rho GTPases. 3-Azibutanol labeled Glu-193, which is located in the vicinity

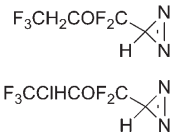

Figure 7. *H*-Diaziflurane (top) and aziisoflurane (bottom).

of, but outside the geranylgeranyl chain binding pocket.[45] Further, it was observed that *N*-acetyl-geranylgeranyl cysteine, a mimic of the geranylgeranylated C-terminal cysteine of Rho GTPases, binds specifically to RhoGDIR and enhances Glu-193 photolabeling by 3-azibutanol.

In studies of L1, a neural cell adhesion molecule whose disruption is implicated in the development of fetal alcohol spectrum disorders (FASD),[44] 3-azibutanol (11 mM), like ethanol, inhibited L1 adhesion in stably transfected NIH/3T3 cells, whereas subanesthetic concentrations of 3-azioctanol (14 μM) antagonized ethanol inhibition of L1 adhesion. 3-Azibutanol and 3-azioctanol were photoincorporated into Tyr-418 on Ig4 and into Glu-33 and Glu-24 to Glu-27, two adjacent regions in the N terminus. A homology model of human L1 Ig1−4 (residues 33−422), based on the structure of the Ig1−4 domains of axonin-1, suggests that Glu-33 and Tyr-418 form a hydrogen bond at the interface of Ig1 and Ig4 to stabilize a horseshoe conformation of L1 that favors homophilic binding. Furthermore, this alcohol binding pocket lies within 7 Å of Leu-120 and Gly-121, residues in which missense mutations can cause neurological disorders that are similar to FASD.

In more complex systems, such as the torpedo nicotinic acetylcholine receptor (nAChR), a ligand-gated ion channel (LGIC), 3-[^{3}H]azioctanol photoincorporated with high efficiency into the α-subunit of the nAChR, with the primary site of incorporation being αGlu-262, within the ion channel at the extracellular end of αM2.[46] Additional incorporation sites are αHis-408 and αCys-412, situated at the lipid−protein interface and αTyr-190 and αTyr-198, at the agonist-binding site. The incorporation in αM4 was independent of the presence of other drugs, while the incorporation at αGlu-262 increased for nAChR in the desensitized state. And incorporation at αTyr-190/αTyr-198 was seen only in the absence of carbamylcholine or α-bungarotoxin, highlighting the usefulness of 3-azioctanol in state-dependent photolabeling of ligand gated ion channels.

3.3. Diazirine Analogs of Inhaled Anesthetics

The diazirine analogs of halothane and isoflurane were synthesized to study inhaled anesthetic binding site and to examine the mechanism of action for volatile anesthetics in proteins.[49,50] *H*-Diaziflurane (Figure 7) is reported to be a nontoxic and potent anesthetic that potentiates GABA-gated ion channels in primary cultures of hippocampal neurons. Calorimetric and structural characterizations have shown that H-diaziflurane binds to the model anesthetic host protein apoferritine with similar energetics as isoflurane. Moreover, it forms photoadducts with residues lining the isoflurane binding site. Analysis with X-ray crystallography and mass spectrometry indicate H-diaziflurane photoincorporates into Ser-27 and Leu-24 of apoferritin.[50] These residues are located in the interhelical dimerization interface between two apoferritine molecules. Leu-24 is also known to be labeled by the diazirine (aromatic) analog of general anesthetic propofol.[51]

A newer version of isoflurane, azi-isoflurane (Figure 7) shows higher potency in tadpoles as compared with isoflurane.[52] Azi-isoflurane labels horse spleen apoferritin at Arg-59 and

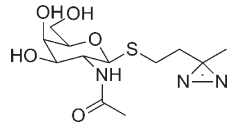

Figure 8. A diazirine analog of sugar 3-azi-1-[([6-3*H*]-2-acetamido-2-deoxy-1-β-D-galactopyranosyl)thio]-butane.

integrin LFA-1 at Tyr-257, as detected by mass spectrometry. These two residues were also detected in the isoflurane binding site in crystallography studies.[53,54]

3.4. Diazirine Analogs of Sugars

UV irradiation of purified glucose transporter GLUT1 in liposomes, in the presence of 3-deoxy-3,3-azi-D-glucopyranose (3-DAG) produced a covalent incorporation of sugar moiety into GLUT1, and 200 mM D-glucose abolished this 3-DAG incorporation. Analyses of trypsin and endoproteinase Lys-C digestion of 3-DAG-photolabeled GLUT1 revealed that the cleavage products corresponding to the residues 115−183, 256−300, and 301−451 of GLUT1 sequence were labeled by 3-DAG. These findings demonstrated that both the C-terminal half and N-terminal regions of the transmembrane domain participated in the putative substrate channel formation.[55] Several derivatives that have the diazirine ring at different positions of the sugar ring have been synthesized, and photolabeling abilities have been tested.[56] The protocol for the synthesis and photo-cross-linking of diazirine-containing sugars has been described recently.[57] Aliphatic diazirine-containing sugars have also been used to covalently trap interactions among glycoproteins by metabolically incorporating these compounds into cell surface glycoproteins.[58] Relative to larger cross-linkers, aliphatic diazirine-containing sugars showed enhanced metabolic incorporation and minimized perturbation. These studies suggest that labels carrying reactive diazirine group directly at the D-glucose moiety of the molecule can be used to label proteins with carbohydrate recognition sites. Similar types of molecules have also been found to have utility in labeling renal brush border membranes and identifying glucose recognition site for Na^+ D- glucose cotransporters.[59,60] A new diazirine analog was synthesized for its use as a donor−acceptor reagent to study a single tryptophan protein using a high-throughput screening method.[61] A further addition to this growing list are the thioglycosides containing an aliphatic diazirine moiety that were synthesized and used for photolabeling of lysosomal 6-hexosaminidase.[62] One of the compounds within this series, 3-azi-1-[([6-^{3}H]-2-acetamido-2-deoxy-1-β-D-galactopyranosyl)thio]-butane (Figure 8) labeled Glu-355 of 6-hexosaminidase B.[63] Glu-355 is highly conserved and located within a region of Hex B that shows considerable homology with the α-subunit of human hexosaminidase A and other hexosaminidases from various species.

3.5. Diazirine Analogs of Etomidate

Etomidates are intravenous general anesthetics known to interact with ion channels. In order to study the anesthetic binding site in nicotinic acetylcholine[18,64] and γ-aminobutyric acid (GABA) receptors,[65] diazirine analogs of etomidate (Figure 9) have been synthesized and photoincorporated into these ion channels. Measurements of the amount of photolabeled residues in the open and desensitized states of the receptors have provided important information on the anesthetic-binding mechanisms in

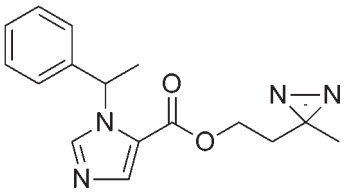

Figure 9. Azietomidate.

these receptors. In nicotinic acetylcholine receptor, UV irradiation resulted in preferential [^{3}H]azietomidate photoincorporation into its α and δ subunits. Within the receptor ion channel in the desensitized state, labeling of αGlu-262 and δGln-276 at the extracellular end and δSer-258 and δSer-262 toward the cytoplasmic end was observed. Within the acetylcholine binding sites, [^{3}H]azietomidate photolabeled αTyr-93, αTyr-190, and αTyr-198 at the $\alpha-\gamma$ interface and δAsp-59.

In the time-resolved photolabeling experiments, the receptor was frozen after 50-ms exposure to agonist and [^{3}H]azietomidate. The amino acids were photolabeled in the ion channel [position M2−20 (αGlu-262, βAsp-268, δGln-276)], δM1 (δCys-236), and αMA/αM4 (αGlu-390, αCys-412), which were also photolabeled in the equilibrium desensitized state of the receptor at approximately half the efficiency. These studies identified etomidate binding sites at the extracellular end of the ion channel, in the delta subunit helix bundle, and in the nAChR cytoplasmic domain that seem similar in structure and accessibility in the open and desensitized states of the nAChR.[64] The M1−M4 helices are the transmembrane domain, and MA is the intracellular domain.

An aromatic diazirine analog of the etomidate exerts a different pharmacology on nicotinic acetylcholine receptor than the azietomidate and binds at a different region in the receptor. The labeled residues were identified as[66] δLeu-265, αVal-255, δVal-269, αSer-252, and γMet-299.

In the $GABA_A$ receptor, [^{3}H]azietomidate photoincorporation is increased by GABA and inhibited by etomidate in a concentration-dependent manner, with an IC_{50} of 30 μM. Two photolabeling sites were detected, one within the M1 transmembrane helix at Met-236, and one within the M3 transmembrane helix at Met-286.[65] The conclusion derived from this study is that the localization of drug binding sites to subunit interfaces may be a feature not only for GABA and benzodiazepines but also for etomidate and other intravenous and volatile anesthetics.

3.6. Diazirine Analogs of Steroids

Diazirine analogs of bile salts 3,3-azi-7α,12α-dihydroxy-5β-[7β-^{3}H]cholan-24-oic acid and (3,3-azi-7α,12α-dihydroxy-5β-[7β-^{3}H]cholan-24-oyl)-2-aminoethane sulfonic acid have been used for photolabeling tissues and cells.[67,68] Several other derivatives of cholesterol-containing aliphatic diazirine groups have also been used to probe cholesterol−protein interactions. 6-Photocholesterol (Figure 10) is one such analog in which the diazirine functionality replaces the 5,6 double bond in the steroid nucleus. This compound has been employed in a diverse number of studies including the identification of cholesterol binding in proteinsin neuroendocrine cells,[69] tracking distribution of and transport of cholesterol in *Caenorhabditis elegans*[70] and probing lipid−protein interactions in oligodendrocytes,[71] SNARE-protein syntaxin,[72] tetraspanins CD9, CD81, and CD82,[73] and the metabotropic glutamate receptor.[74] Another derivative [^{3}H]-25OH-6-azi-cholestanol was used to study oxysterol-binding protein-related proteins,[75] E1 fusion protein from Semliki Forest

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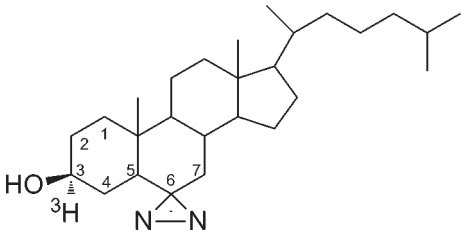

Figure 10. 6-Photocholesterol.

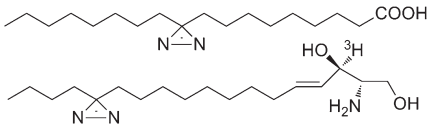

Figure 11. Diazirine analogs of lipids, 10-azistearic acid (top) and D-*erythro*-photospingosine (bottom).

virus (SFV),[76] and cholesterol homeostasis.[77] A photoactivatable cholesterol analog, 7-azicholestanol, was introduced by Cruz et al.,[78] where the diazirine group was attached to the steroid moiety of cholesterol at position number 7. This analog compound was used to label the Niemann−Pick type C1 (NPC1) protein[79] and the steroidogenic acute regulatory lipid-transfer domain-containing proteins StAR and MLN64.[80] Using positional isomers 3-azi- and 7-azi-cholyltaurine, Kramers et al. identified a tripeptide as the possible bile acid binding site in ileal lipid-binding protein (ILBP).[81] In order to study the molecular mechanism of $GABA_A$ receptor modulation by neuroactive steroids, a new diazirine analog, 3α,5β-6-azi-3-hydroxypregnan-20-one (6-AziP), was developed where the diazirine moiety is attached to the steroid moiety at position 6. Using this molecule, the protein VDAC-1 was detected as a neuroactive steroid binding protein that may modulate the function of $GABA_A$ receptor.[82,83]

Blanton's group[84] identified [3α-^{3}H]6-azi-5α-cholestan-3β-ol (^{3}H-azicholesterol) photolabeled residues, by Edman sequencing, while studying the role of cholesterol in the activity of acetylcholine receptor. [^{3}H]Azicholesterol partitioned into nAChR-enriched membranes very efficiently (>98%) and photoincorporated into nAChR subunits on an equal molar basis. Photolabeled amino acid residues in each M4 segment correspond to acidic residues located at either end of each transmembrane helix (e.g., αAsp-407). This study established that cholesterol interacts with M4, M3, and M1 segments of each subunit, and the interaction is highest in the M4 segment; the cholesterol binding domain fully overlaps the lipid−protein interface of the nAChR.[84]

3.7. Diazirine Analogs of Lipids

The synthesis and photochemical properties of the first aliphatic diazirine-based phospholipid were reported by the Khorana group.[24] Photolysis of this aliphatic diazirine analog of phospholipids in vesicles did not photolabel the membranes. These findings were explained on the basis of the photochemistry of tetrafluorodialkyldiazirines that did not yield insertion product but rearranged internally by alkyl group migration to produce olefin and underwent intramolecular insertion reaction generating cyclopropanes.[24] The aromatic analogs, on the other hand, labeled proteins[85−89] and for the transmembrane domain of glycophorin A, labeling was found to be at the Glu-70[90] site.

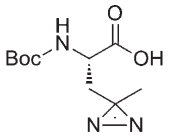

Figure 12. Diazirine analog of an amino acid.

The photoactive fatty acid 10-azistearic acid (Figure 11), another diazirine derivative, serves in combination with [^{3}H]choline or [^{3}H]inositol as a tool to probe protein−PC and protein−PI interactions, respectively.[69] A novel photoactive sphingolipid has also been synthesized to study protein−lipid interactions in the ceramide transporter protein allowing the detection of protein−sphingolipid interactions within the membrane bilayer of living cells.[91] This radioactive and photoactive sphingosine ([^{3}H]-D-*erythro*-photosphingosine) (Figure 11) circumvents the detection of non-sphingolipid-interacting proteins upon degradation.[91]

3.8. Diazirine Analogs of Amino Acids

Several diazirine analogs of amino acids have been incorporated into peptides in order to study basic protein−protein interactions. The diazirine analog of methionine, photo-Met, was used to transiently capture MH2−MH2 domain interaction in proteins, further demonstrating that this amino acid is compatible with solid-phase peptide synthesis and expressed protein ligation.[92]

In another study, a photoaffinity probe with a diazirine moiety attached to leucine (Figure 12) was used for photo-cross-linking proteins under native conditions.[93] The diazirine-containing amino acids photoleucine and photomethionine bypassed the proofreading machinery when loaded onto tRNA, demonstrating the biocompatibility of the photoactivatable diazirine group.[94]

3.9. Diazirine Analogs of Nucleic Acids

Aliphatic diazirine-based DNA photo-cross-linking probes (Figure 13) have been developed for the study of protein−DNA interactions.[95] This probe has an aliphatic diazirine moiety attached to DNA molecules, which photo-cross-linked its interacting proteins.

3.10. Miscellaneous

A cinnamoyl derivative, 3-(3-methyl-3*H*-diazirin-3-yl)-*N*-{4-[(*E*)-3-oxo-3-(pyridin-3-yl)prop-1-enyl]phenyl}propanamide (Figure 14), was synthesized, and photolabeling studies were performed on guinea pig liver transglutaminase.[17] Mass spectrometric analysis identified Cys-230 as the photolabeled residue, which is somewhat solvent exposed in the "closed" conformation as compared with the open conformation of the receptor. The labeled residue is located very close to the catalytic site, thereby explaining the mode of inhibition for these derivatives.

Diazirine analog of hexerestrol derivative (Figure 14) was synthesized and used for estrogen receptor labeling.[96,97] Several fluorescent molecules, such as acridine, indocarbocyanine, and styrene, were modified via attachment of an aliphatic diazirine group and used for selective photoaffinity labeling of a protein in *Saccaromyces cerevisiae*.[98] Recently the binding site of Delta (lac) acetogenine in bovine heart mitocondrial NADH−ubiquinone oxidoreductase has been investigated using its aliphatic diazirine derivative.[99]

4. DETECTION OF PHOTOLABELING AND IDENTIFICATION OF THE PHOTOLABELED RESIDUES

The diazirine moiety absorbs light in the range of 300−380 nm, depending on the nature of the molecule to which it is attached. In a typical photolabeling experiment, the protein and the diazirine compound are incubated in an aqueous buffer for a defined length of time to allow the ligand to diffuse into the binding site and then photolyzed in an inert atmosphere using lamps that generate 300−380 nm light. The photolabeling efficiency can vary depending on the intensity of the lamp and the buffer used for photolabeling.[43,100] The half-life ($T_{1/2}$) of photolysis is related to the intensity of the lamp (I_0), molar extinction coefficient (ε), and quantum yield of photolysis (Ψ) according to the following equation:[101]

$$T_{1/2} = 0.3/(\Psi I_0 \varepsilon) \quad (1)$$

Traditionally, protein labeling is detected by measuring the incorporation of radiolabeled ligands into the protein after photoirradiation. The percent of incorporation can be calculated from the specific activity of the radioligand. For detecting radiolabeled residues, photolabeled protein is digested with suitable protease and the peptides are sequenced using the Edman degradation method. This method uses the reaction of phenylisothiocyanate (PITC) with the amino group of the N-terminal amino acid, followed by cleavage, conversion to a phenylthiohydantoin (PTH) derivative, and detection[102−104] by HPLC analysis. Additional step(s) of further protease digestion and HPLC analysis may be employed (Figure 15), depending on the size and characteristics of the protein (soluble or membrane bound). For complex membrane bound receptors consisting of several subunits, additional steps of acetone precipitation and separation using gel electrophoresis are necessary.[18,105−107]

In nonradioactive methods, photoincorporation can be detected either by MALDI-TOF or ESI mass spectrometery.[81,108−110] While in MALDI-TOF, new peak(s) for the photolabeled protein appear directly in the chromatogram, deconvolution of different charge states generates new peaks in an ESI mass spectrometer (Figure 15). Both mass spectrometric techniques measure the stoichiometry of photoincorporation and can be conveniently used for relatively smaller sized proteins and also when the photolabeling efficiency is above the detection limit of the mass spectrometer. In some cases, the higher efficiency of photolabeling and the increased

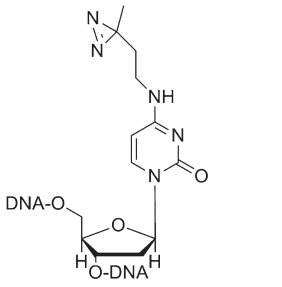

Figure 13. Diazirine analog of a nucleotide.

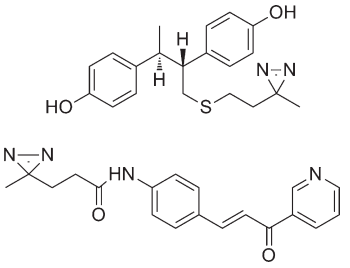

Figure 14. Diazirine analogs of cinnamic acid (top) and hexerestrol (bottom).

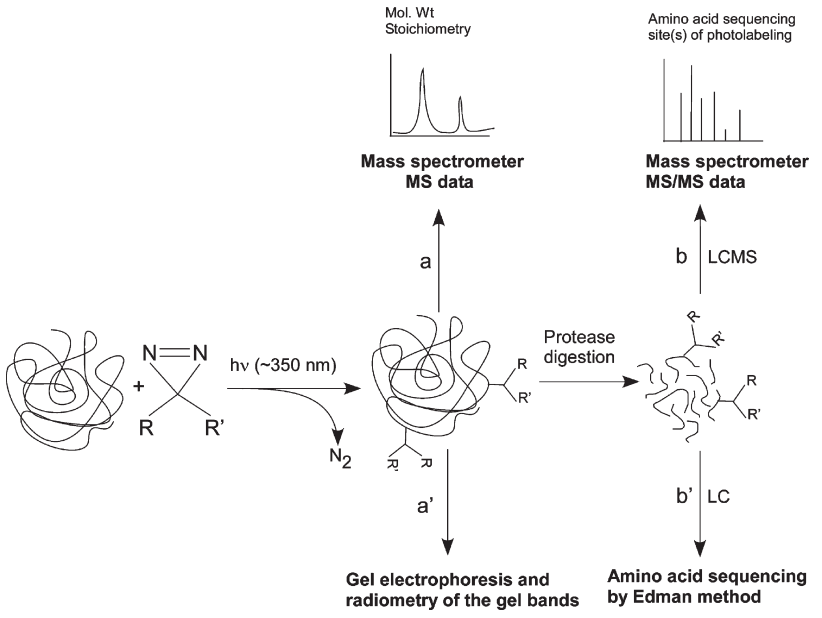

Figure 15. Simplified scheme for the detection and analysis of photolabeling. In the nonradioactive mass spectrometric method, the photolabeld protein can be analyzed either by MALDI-TOF or by ESI mass spectrometer to identify photoincoporation and stoichiometry (a); the sites of photoincorporation are detected by acquiring MS/MS data for the digested sample in ESI mass spectrometer (b). In the radioactive method, radiolabeled diazirine is used for photolabeling agent. Detection of photoincorporations is done by running the radiometric detection of protein from the gel (a′); amino acid analysis is done with the digested sample using Edman method (b′). For membrane bound proteins, additional steps of removing the membrane are employed before the digestion process.

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hydrophobicity of the photolabeled products compared with the original protein lead to the isolation of the photolabeled product by both capillary and regular column in liquid chromatography. For example, photoadducts of 3-azioctanol and PKCδC1A, PKCεC1B, or Munc 13.1C1 were separated from the unlabeled protein and characterized by mass spectrometry.[15,48]

For detecting the photolabeled residues, the protein is usually digested with protease(s), and the peptides are detected by measuring their masses. For cysteine-containing proteins, additional steps of reduction of disulfide bonds with DTT and alkylation of cysteines by alkylating agents, such as iodoacetamide or 4-vinyl pyridine, before protease digestion are necessary to circumvent the problem of disulfide bond formation among the peptides and possible aggregation. For the peptide sequencing second (MS/MS) or higher order mass data[13−17] are acquired. The first step in the most popular liquid chromatography mass spectrometric (LC-MS) sequencing using an electrospray ion source is the identification of the photolabeled peptide. This is followed by fragmenting the peptide under mass spectrometric conditions to generate fragment ions such as a, b, c, x, y, and z.[111] The a, b, and c ions describe bond-cleavage fragments that retain the amino, that is, the N-terminal side of the peptide, and the x, y, z ions describe bond-cleavage fragments that retain the carboxyl, that is, C-terminal side of the peptide.[111] Usually, for peptide sequencing, the electrospray source is combined with an ion trap, quadrupole mass filter, or time-of-flight mass analyzer.

Recently orbitrap and the FT-ICR[112,113] techniques have been shown to provide very high mass accuracy, high resolution, and high dynamic range detection. In the normal mass spectrometric condition, the b and the y ions predominate and the matching is usually performed by mass spectrometry based software programs such as SEQUEST, MS-Tag, PeptideSearch, etc. In the software program, the label (diazirine or other modification) is placed on each peptide residue to obtain the predicted fragment ion masses that subsequently are matched with the experimentally measured fragment ions. A library of the predicted peptides and their fragmentation pattern for a particular protease can be generated if the primary sequence of a protein is known. The experimental data can then be searched against this library. In addition, although more time-consuming, the experimental data can be searched against the entire database of peptides for that particular species. In the SEQUEST algorithm, mass accuracies, Xcorr (cross correlation) or dCn (Delta correlation), are key parameters important in identifying the best peptide match. The number one hit will always have the highest value of Xcorr, because Xcorr is used to produce the final ranking of the candidate peptides in the search. Xcorr values are usually higher for well-matched, large peptides, and lower for smaller peptides, although there is no absolute value to judge how good a match is for a peptide. The magnitude depends on the size and charge of the peptide. For example, higher molecular weight and higher charge state peptides generate higher Xcorr values. The dCn values are calculated from Xcorr values, and higher values indicate a better match. The dCn value represents how different the first hit is from the second hit in the search results. A general rule of thumb is that a dCn of 0.1 or greater is acceptable. For a large database (nr.fasta or owl.fasta), dCn values are smaller than those obtained from a small database, primarily because the probability of sequence similarity is higher in the larger databases.

The methodology described above was used successfully to detect 3-azioctanol photoincorporation and identification of the labeled residue in PKCεC1B.[14] When the photolabeled protein was run through the LC-MS using a C18 capillary column, the major portion of the photolabeled protein appeared at a different retention time compared with the unlabeled protein in the liquid chromatogram. The charge envelope for the labeled protein at this retention time is shown in (Figure 16A,B). The deconvolution of this charge envelope using a mass spectrometry based software (Bioworks Browser from ThermoFisher) generated three peaks at 7464 for the unlabeled protein, 7592 for labeled protein with 1:1 stoichiometry, and 7720 for the labeled protein with 1:2 stoichiometry. Each molecule of 3-azioctanol added up 128 Da after photolysis. It is also possible to detect photoincorporation without separating the labeled and unlabeled proteins by liquid chromatography. The mass peak of each charge state for the photolabeled protein is close to the peak for the same charge state of the unlabeled protein because the molecular weight of the label is much less compared with that of the protein. Consequently, there are two different charge envelope deconvolutions for labeled and unlabeled proteins.

To detect the photolabeled residue, second or higher order mass data are acquired for a peptide in an ESI mass spectrometer. Figure 16C shows the MS/MS data for a labeled peptide generated from trypsin digestion of the 3-azioctanol labeled PKCεC1B. All possible peptides from the digest were detected by matching corresponding b and y ions. A doubly charged peptide with m/z (mass/charge) of 504 in the chromatogram corresponds to the molecular weight of FGIHNYK plus 128 Da indicating that the peptide was modified by one molecule of 3-azioctanol. To determine the site of labeling, the label (128 Da) was placed on each residue of the peptide and mass values of corresponding b and y ions were calculated using SEQUEST. The observed MS/MS spectrum was matched with the calculated mass values. Tyrosine (Y) was found to be the labeled residue because all the b ions starting from Y and higher and all the y ions starting from Y and higher had additional mass of 128 Da.

The principle behind the identification of the diazirine modification is more or less similar to the mass spectrometric identification of post-translational modifications such as phosphorylation and glycosylation,[111,114] which warrants for a detailed discussion and outside the scope of this review.

One of the most important aspects of receptor photolabeling is the quantitative determination of photolabeled residues. Typically, quantitative measurements are made using the radiolabeled diazirine compounds following the Edman degradation method. In the final step of measuring PTH derivatives, one measured fraction goes to the PTH analyzer, and the other part goes to the scintillation counter for measuring radioactivity.[18,46,65] Using this methodology for quantitation of azietomidate labeling, researchers found that in acetylcholine receptor αGlu-262 was labeled with 10-fold higher efficiency than δGln-276 and δAsp-59[18] and in GABA receptor Met-236 labeling was higher than that of Met-286.[65] While the relative efficiency of photolabeling can be determined fairly accurately with the radioactive diazirine compounds and Edman method, quantitative measurements of mass spectrometric methods are still under development. The relative photolabeling efficiency of residues can be determined by mass spectrometry by comparing the relative ion intensity of the labeled and the unlabeled peptides. In this case, however, two assumptions are necessary: (1) the peptide should be a singly modified peptide, and (2) the ionization efficiency of the labeled and the unlabeled peptides must be the same. Usually when the labels are uncharged and contain aliphatic chain, the ionization efficiencies of labeled and unlabeled peptides could be almost the same. For absolute

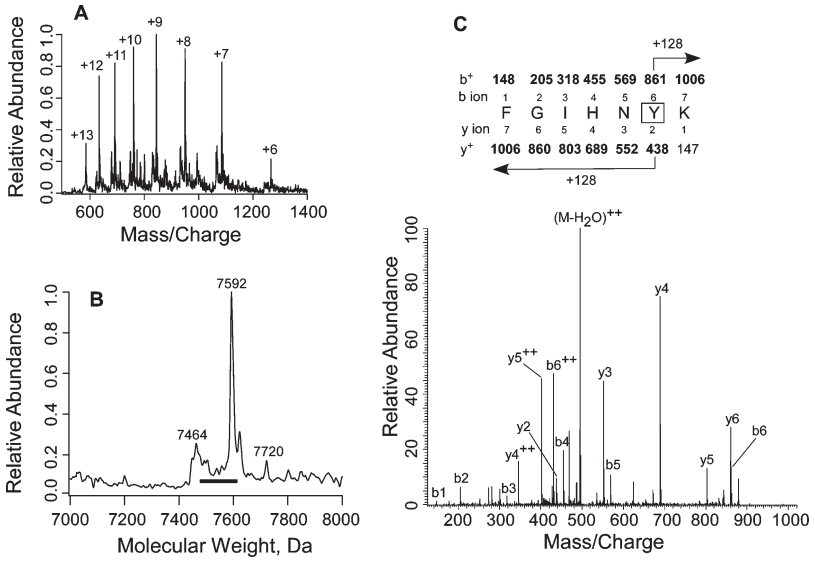

Figure 16. Mass spectral identification of the photoincorporation of azioctanol into PKCεC1B: (A) Charge envelope of the photolabeled PKCεC1B obtained after the sample was infused into the LTQ linear ion trap mass spectrometer (Thermo Fisher). (B) Deconvoluted mass spectrum of the charge envelope of PKCεC1B photolabeled with 1 mM 3-azioctanol. Bar shows mass shifts of 128 Da corresponding to the incorporation of a single molecule of 3-azioctanol into PKCεC1B. A small peak at 7720 Da corresponds to the incorporation of two azioctanol molecules. (C) Identification of Tyr-250 as the site for 3-azioctanol. MS/MS data for the 3-azioctanol (1 mM)-modified heptamer peptide FGIHNYK. At the top of the figure, the predicted charge/mass ratio of N-terminal ions (b ions) and C-terminal ions (y ions) are shown above and below the sequence, respectively. They are singly charged unless noted otherwise. The horizontal arrows show which m/z values for the b ions (above) and y ions (below) have a mass of 128 Da for 3-azioctanol added to them. Observed values are shown in bold. In addition to the singly charged ions, three doubly charged $y4^{+2}$ (m/z 345), $y5^{+2}$ (m/z 402), and $y6^{+2}$ (m/z 431) ions are also observed.

quantitative measurements by mass spectroscopy, an isotopically labeled (usually ^{13}C or ^{15}N) internal standard peptide is spiked into the peptide digest and analyzed simultaneously in the same run. To quantify the photolabeled peptide, the standard peptide should contain both the photolabel and ^{13}C or ^{15}N. The same principle is followed for the identification and quantification of protein post-translational modifications (phosphorylation, acetylation, methylation, etc.).[115−119] To use as a standard, a peptide modified with phosphate, acetyl or methyl groups can be synthesized without problems, while the synthesis of a peptide with a photolabel and isotope label may require additional synthetic ingenuity. For studying photolabeling in the presence of effectors (activator/inhibitor) and quantification of photolabeling, use of a ^{13}C-labeled diazirine may be essential. Two samples, one labeled in the presence of regular diazirine (^{12}C) and the other in the presence of ^{13}C diazirine, together with the effector, are mixed in equal amounts and analyzed simultaneously. The intensities of these two peaks are proportional to the relative probability of photoincorporation and, hence, also proportional to the affinity. The quantitative ratios can be measured either from a relative comparison of signal intensities within a single MS scan or with peak areas determined from extracted ion chromatograms of the heavy and light photolabeled peptide.

The advantages of mass spectrometric analysis over conventional Edman methods are several. Namely, it is fast, can detect a peptide at a much lower concentration, uses a milder detection condition, and does not require radioactive ligands. Edman methods of peptide sequencing are difficult if the stoichiometry of labeling is low and may be blocked at the modified site. Further, if the N terminal of a peptide is blocked due to post-translational modifications of proteins such as glycosidation, acetylation, phosphorylation, and fatty acid addition, the Edman method cannot be applied. Sometimes extraction of the labeled peptides from the gel also becomes difficult because of these modifications. As mentioned earlier in reference to the characteristics of an ideal photolabel, the photolabel should survive the conditions used for detection. In the Edman degradation sequencing conditions, the label can be dissociated from the peptide by elimination/hydrolysis as predicted for 3-aryl-3-(trifluoromethyl) diazirine.[120] It was observed that in the photolabeling of acetylcholine receptor with radioactive 3-azioctanol, the residue modified by 3-azioctanol was labile under HPLC condition.[46] In another instance, use of 25% aqueous trifluoroacetic acid for 20 min at 64 °C in the Edman sequencing methods could not identify the labeled residue Glu-355. However, by changing the condition to 25% aqueous ammonium hydroxide, the residue was converted to Gln-355 and was detected successfully.[63]

Although mass spectrometric analysis has several advantages over traditional Edman degradation methods, it is not completely flawless, particularly when it comes to the identification of labeled residues, which relies totally on the accurate interpretation of the product ion spectra. As of now, the major limitation of mass spectrometry probably is that the methods for quantitative measurements of the labeled residues are not well-developed.

5. AMINO ACIDS LABELED BY ALIPHATIC DIAZIRINES

Like most photolabeling agents, the reaction rate of the photointermediates produced from aliphatic diazirines with a given side chain is expected to vary. The protein residues labeled by aliphatic diazirines are listed in Table 1. These residues have been identified either by mass spectrometric sequencing or by classical Edman degradation methods. It is evident from the table

that aliphatic diazirines exhibit a broad range of side chain reactivity and can react with nucleophilic side chains such as aspartate and glutamate, positively charged side chains such as histidine, lysine, and arginine, and neutral side chains such as tyrosine, methionine, serine, and cysteine. In several studies, where the photolabeling was exclusively on a residue having an acidic side chain, the pertinent concern was whether the labeling was a true reflection of the close proximity of diazirines to the ligand binding residues or of simply a mechanism of side chain reactivity. For example, in the photolabeling study of the acetylcholine receptor and photocholesterol,[121] the conserved aspartates at the N-terminus of each M4 segment (Asp-407, Asp-436, Asp-448, and Asp-454) were labeled. The only acidic side chain at the C-terminus of the M4 segments, Asp-457, also was labeled. However, the authors argued against this being a preference of aliphatic diazirine for the acidic chains, because, another aliphatic diazirine, [^{3}H]Azioctanol incorporated into His-408 and Cys-412 within the RM4 segment, but there was no reaction with Asp-407.[46] Later, in the case of another aliphatic diazirine, azietomidate labeling to acetylcholine receptor, tyrosine, glutamine, serine, cysteine, and histidine also were labeled along with aspartate, glutamate, and tyrosine.[18,64]

Tyr-236, Tyr-250, and Glu-582 are the preferred photolabeling sites of azialcohols in PKCδC1B,[13] PKCεC1B,[14] and Munc 13.1C1,[48] respectively, although there are several tyrosine and glutamate residues in these proteins. To further investigate and track down the most favored site, the strategy of titrating the photolabel concentration to eliminate nonspecific photoincorporation was adopted and was shown to be essential and effective.[14,15,45] In the case of PKCδC1B, 1 mM azioctanol labeled Tyr-236, but no photolabeling was observed at Tyr-238 even after the concentration of azioctanol was raised to 10 mM. Despite these two tyrosine residues being one residue apart from each other, one showed preference over the other. Therefore, the fact that in many cases reactive residues that were in the vicinity of the binding site but not actually in it (based on independent structural information) were not photolabeled suggests that photolabeling is indicative of occupancy first and of reactivity next. With radiolabeled diazirines, the relative amount of photoincorporation was determined at the residue level,[18,46,65] but it is difficult to know how much of it was contributed by the side chain preference in the absence of a systematic study on the relative efficiency of the reaction of the amino acid residues with an aliphatic diazirine.

The relative preference of the aromatic diazirine[122] and benzophenone[123] for amino acids in vitro has been reported. The aromatic diazirine, 3-(trifluoromethyl)-3-(*m*-iodophenyl) diazirine (TID) was immobilized on a glass surface and its relative reactivity with amino acids revealed the reactivity scale where cysteine was at the top and glycine was at the bottom of the scale. The top ten labeled residues were Cys > Trp > Tyr > His > Phe > Thr > Met > Ser > Pro > Ile. The bottom ten residues were Asp > Leu > Asn > Lys > Val > Ala > Arg > Glu > Gln > Gly. Although the amino acids could have been immobilized to mimic the protein environments, this study provided useful information on the relative reactivity of amino acids in a defined condition. To justify their experimental observations the authors argued that with several exceptions, aromatic diazirines labeled methionine, cysteine, phenylalanine, glutamate, tryptophan, etc. in several proteins.[90,124−129]

Do diazirines have preferences for particular functional groups or for a specific chemical bond of an amino acid for labeling? Out of the two reactive species of diazirine photolysis, singlet carbene and diazo compounds, singlet carbenes are known to undergo various types of intermolecular reactions such as insertion to the C−H, O−H, and C═C bonds, reactions with nucleophilic groups, proton abstraction, etc., and intramolecular reactions such as rearrangements to olefins, dimerization, and intramolecular insertion reactions. While there is no report of a systematic study on the functional group preference for carbenes either in solution or in protein environment, several related studies in solution indicated that the reactivity of a carbene depends largely on substituent groups. For example, photoregenerated singlet methylene reacts with nucleophiles more rapidly than insertion into the C−H bond,[130] whereas difluorocarbene (:CF2) is highly selective for alkenes substituted with different groups and does not insert into C−H bonds.[131] The Khorana group characterized a labeled peptide where the carbene inserted into the carboxylic group of Glu-70[90] in glycophorin A. The other photointermediate (the diazo compound) generates carbene under photolytic conditions or can generate a carbocation that reacts with the nucleophilic heteroatoms of the amino acid. From combined mass spectrometry and crystallographic data analysis for the aziisoflurane labeling to apoferritin, it was proposed that backbone carbonyl oxygen also could be a target for diazirines.[52]

6. NONSPECIFIC LABELING AND THE USE OF SCAVENGERS

Any discussion of photolabeling would be incomplete without mentioning the nonspecific labeling that occurs in almost every photolabeling experiment. Nonspecific labeling can occur in two ways. First, if the concentration of the ligand in solution is high compared with the protein, the reactive species generated in solution may react on the surface of the protein generating nonspecific labeling. Second, if the lifetime of the reactive intermediate is long relative to diffusion processes, nonspecific labeling may occur even at higher protein concentrations. To minimize this effect, several investigators used radical scavengers[132] that can react with the reactive intermediate generated at the surface or the long-lived intermediate outside of the binding pocket. Scavengers were used for several photoreagents including adamantyl diazirine[42] and an aromatic diazirine analog of nicotinamide adenine dinucleotide.[133] Small molecules such as *p*-amino benzoic acid, *p*-aminophenylalanine, tris, reduced glutathione, and β-mercaptoethanol and protein such as bovine serum albumin (BSA) also can be used as scavengers.[132] Molecular oxygen can also act as scavenger by reacting with carbene. Ideally, the scavengers do not absorb light and do not bind with the receptor. In general, the nonspecific labeling can be reduced by designing high-affinity ligands and adjusting the protein and receptor concentration to maximize the ratio of bound and free ligand.

7. PERSPECTIVES

The objective of this review is to present an unbiased overview of the advantages and disadvantages of aliphatic diazirine and help the readers evaluate the effectiveness of aliphatic diazirines as photolabeling agents. From an extensive literature review conducted herein, it is evident that more and more researchers are using aliphatic diazirines for photolabeling studies despite initial apprehension. Many of these investigations identified photolabeled residues on the ligand binding site of soluble as

well as membrane proteins. One of the ways to validate photolabeling results is to determine the role of photolabeled residues in ligand binding and in modulating the function of proteins.

To determine functional significance of photolabeled residues, a common practice is to mutate them and examine the effects of these mutations on function/activity of the protein. In the photolabeling study of PKCεC1B by azialcohols, the photolabeled residues His-248 and Tyr-250 were mutated and the mutant H248A/Y250A showed reduced alcohol binding, both in terms of photolabeling and its allosteric alcohol binding.[14] In the study of acetylcholine receptor, photoactivated azioctanol irreversibly desensitized the receptor via interactions at the photolabeled residue αGlu-262.[47] In $GABA_A$ receptor, mutation of the etomidate labeling sites Met-236 and Met-286 to the corresponding tryptophan residues on α1 or β2 subunits enhanced $GABA_A$ receptor gating and reduced etomidate modulation.[134] It was also observed that the threonine residue next to Met-236 had the highest effect on the $GABA_A$ activation with neurosteroids.[135] When the azialcohol photolabeled residues Glu-33 and Tyr-418 were mutated to cysteines in Glu33Cys/Tyr418Cys of L1, adhesion was found to be significantly higher and the efficacy and potency of alcohol was significantly lower than the wild-type L1.[136]

That the photolabeled residues represent the true binding site is also evidenced from the X-ray crystallographic studies. In the alcohol−PKCδC1B complex, the alcohol molecule forms hydrogen bonds with the photolabeled residue Tyr-236 and is involved in van der Waals interaction with the adjacent Met-239 in the alcohol-binding pocket. When Tyr-236 was replaced by Phe-236 that lacks hydrogen bond forming ability, no alcohol molecule was bound in the putative alcohol binding site.[137] In the aziflurane−ferritine system, the photolabeled residues were found to be at the aziflurane binding site in the crystal structure of aziflurane−ferritine complex.[50,137] Similarly in the case of aziisoflurane−apoferritin and aziisoflurane−integrin LFA1 complexes, the photolabeled residues also are located in the crystallographically defined isofurane binding site.[52] A similar study using an aromatic diazirine analog of retinal identified Trp-265 as one of the residues that form the retinal binding site of rhodopsin.[129] Later, the crystal structure of rhodopsin[138] confirmed the presence of Trp-265 in the retinal binding pocket.

Although there have been significant advances in the use of aliphatic diazirine during the last 10 years, additional studies are required to tap into the full potential of aliphatic diazirines as photolabeling agents. Identification of the photointermediates of an aliphatic diazirine in a protein environment would be intriguing. A systematic analysis of the relative reactivity of the 20 amino acids is imperative in both homogeneous media including buffers as well as organized constrained media including proteins. The chemical structure of the insertion product of amino acid and the diazirine would provide insight on the reactivity of a particular chemical bond in the amino acid residue. Finally, although challenging and potentially contradictory in the sense that photolabeling is an alternative to ligand binding site structure, the photolabeling results must be validated by structural studies wherever possible. The notion that the diazo compounds diffuse out of the ligand binding site to label other residues and that the aliphatic diazirines photolabel acidic residues is not a general one and has not been proven experimentally for all aliphatic diazirines. Nevertheless, pending results of these experiments, it is important to cautiously interpret photolabeling data, considering its possible side chain preferences.

AUTHOR INFORMATION

Corresponding Author

*Tel: 713-743-1708. Fax: 713-743-1884. E-mail: jdas@uh.edu.

BIOGRAPHY

Joydip Das is an Assistant Professor at the Department of Pharmacological and Pharmaceutical Sciences, University of Houston. He received his B.Sc. (Honors) in chemistry from the Presidency College, Kolkata, and M.Sc. in organic chemistry from the Burdwan University, West Bengal. After receiving his Ph.D. from the Indian Institute of Technology, Bombay, in the laboratory of Anil K. Singh, he came to the United States and worked with Dr. Rosalie K. Crouch at the Medical University of South Carolina, with Har Govind Khorana at the Massachusetts Institute of Technology, and with Keith W. Miller at the Massachusetts General Hospital. In 2004, he was appointed as Instructor at the Harvard Medical School. The major focus of his research has been the ligand−protein interaction in soluble and membrane proteins. His currents research goals are to investigate the roles of protein kinase C's in modulating alcohol actions and developing isotype-specific modulator for this family of proteins.

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ABBREVIATIONS

RIES	rearrangement in the excited state
PKC	protein kinase C
LGIC	ligand-gated ion channel
FASD	fetal alcohol spectrum disorders
nAChR	nicotinic acetylcholine receptor
GABA	γ-amino butyric acid
MALDI-TOF	matrix-assisted laser desorption ionization-time-of-flight
ESI	electrospray ionization
DTT	dithiothreitol
HPLC	high-performance liquid chromatography
PITC	phenylisothiocyanate
PTH	phenylthiohydantoin
Xcorr	cross correlation
dCn	delta correlation
TID	3-(trifluoromethyl-3-(m-iodophenyl) diazirine
IC_{50}	inhibitory concentration at half-maximum

PC	phosphatidylcholine
PI	phosphatidylinositol

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