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Identification of alcohol binding site(s) in proteins using diazirine-based photoaffinity labeling and mass spectrometry

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Abstract

Defining molecular targets of alcohol and understanding the molecular mechanism of alcohol actions are necessary to develop effective therapeutics for Alcohol Use Disorder (AUD). Here we describe a detailed protocol for identifying alcohol binding site(s) in proteins using diazirine-based azialcohol as photoaffinity labeling agents. Upon photoirradiation, azialcohol photoincorporates into alcohol-binding proteins. The stoichiometry and site of azialcohol photoincorporation can be determined using high resolution mass spectrometry. Identification of the alcohol-binding residues in protein followed by measuring the biological significance of these residues in regulating alcohol action are important steps in characterizing the molecular targets of alcohol.

Graphical abstract

Keywords

Photoaffinity labeling; diazirine; azialcohol; mass spectrometry; alcoholism

1. Introduction

Alcohol (ethanol) addiction is a global health problem that causes huge number of deaths and enormous economic burden to the society. In the United States alone, excessive alcohol consumption caused an average of 88,000 deaths per year and a cost of \$249 billion to its economy in 2010 [1]. Despite huge loss of lives and economic burden to the society, there are only three FDA-approved drugs in the market to treat alcohol addiction-disulfiram, naltrexone and acamprosate. These drugs have compliance issues and have multiple side effects[2]. Therefore, there is an urgent need for the discovery of new drugs to treat alcohol addiction. Further, to develop newer drugs with minimum side effects, it is imperative to

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Conflict of interest

The author declares no conflicts of interest with the contents of this article.

understand the molecular mechanism of alcohol action and define molecular targets (receptors, enzymes and ion channels) of ethanol in the brain, so that drugs can be designed based on the alcohol binding site(s). Ethanol has multiple targets and affects many neuronal circuitries in the brain[3]. For example, ion channels such as, GABA_A [4], glycine [5] and glutamate [6]; enzymes such as, adenylyl cyclase [7], phosphodiesterase type 4 [8] protein kinase C (PKC) [9], etc. are some of the well-characterized targets of ethanol.

Ethanol contains a polar hydroxyl group which is capable of forming hydrogen bonds and the -CH₂CH₃ group which exerts weak hydrophobic interactions. Therefore, ethanol is both a polar molecule as well as somewhat lipophilic. Ethanol crosses the blood-brain-barrier very easily to reach to the brain. There is now abundant evidence that ethanol can bind to proteins and change their functions [10]. However, the binding energy is only about 1 kcal/mol, meaning that the effect of ethanol binding on a protein will be only slightly more than that of thermal energy (0.6 kcal/mol at room temperature). This low affinity interactions with proteins is consistent with in vivo potency of 10–100 mM [11].

Photoaffinity labeling using diazirine based azialcohols (Figure 1) is an important tool to identify alcohol binding site(s) in proteins leading to defining molecular targets of ethanol. Although aziethanol is the most appropriate photolabel to study alcohol-binding site(s), it has never been synthesized because of the inherent instability associated with the molecule. A possible degradation pathway of aziethanol is shown in Figure 2. The hydroxyl group oxygen can attack the carbon atom of the diazirine ring and form an epoxide. In the presence of water molecule the epoxide can be opened up to form ethylene glycol. However, azibutanol acts as a surrogate for aziethanol. Moreover, two positional isomers of azioctanol, 3-azioctanol and 7-azioctanol have also been used to delineate alcohol binding site(s) in proteins. Thus far these photolabels have been used for detecting alcohol binding sites in PKC δ [12, 13], PKC ϵ [14, 15], Munc13–1[16], nicotinic acetylcholine receptor[17], adhesion molecule L1[18], adenylyl kinase [19] and RhoGTPase[20].

In this article a detailed protocol for photoaffinity labeling of proteins using the diazirine-based azialcohol followed by mass spectrometric identification of the alcohol binding site(s) have been described. To aid to better understanding the principle and concept, the photolabeling data for the C1 domain of a presynaptic protein, Munc13–1 are presented. Recent studies suggest that Munc13–1 is an important target of alcohol [16, 21].

2. Principles of diazirine-based photoaffinity labeling and mass spectrometry

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 with 300–380 nm light. Diazirines generate carbenes, which in most cases undergo insertion into nearby chemical bonds resulting in photolabeling [22, 23]. Carbenes are highly reactive species with lifetimes in the range of nanoseconds. In addition to generating carbenes, diazirines undergo isomerization generating linear diazo compounds (Figure 3). 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 stoichiometry and the site(s) of photoincorporation can be detected by electrospray ionization (ESI) mass spectrometer. Infusion of photolabeled protein into the mass spectrometer yields several charge states for both labeled and unlabeled proteins. Deconvolution of different charge states generates new peaks in an ESI mass spectrometer (Figure 4). As an example, the stoichiometry of 3-azibutanol photoincorporation into the C1 domain of a presynaptic protein Munc13–1 is shown in Figure 5.

For identifying the photolabeled residues, the protein is usually digested with protease(s), and the peptides are separated by liquid chromatography followed by the identification of the photolabeled peptides. For cysteine-containing proteins, additional steps of reduction of disulfide bonds with DTT and alkylation of cysteines by alkylating agents, such as iodoacetamide, before protease digestion are necessary to circumvent the problem of disulfide bond formation among the peptides and possible aggregation.

The photolabeled peptide is then fragmented under mass spectrometric conditions and second (MS/MS) or higher order mass data are acquired. Fragmentation of the peptide generates fragment ions such as a, b, c, x, y, and z. 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. Usually, for peptide sequencing, the electrospray source is combined with an ion-trap, quadrupole mass filter, or time-of-flight mass analyzer. Appropriate softwares such as Xcalibur and SEQUEST can be used to analyze the MS/MS data for the identification of the azialcohol-labeled residue. As an example, the MS/MS data for the photoincorporation of 3-azibutanol into the Glu-582 of a 16-mer peptide derived from the C1 domain of Munc13–1 is shown in Figure 6. 3-Aziocanol also photoincorporates into this residue [16]. The location of this residue in the three-dimensional structure of Munc13–1 C1 shown in Figure 7.

3. Materials

3.1. Reagents

1. Azialcohols- The chemical synthesis of these compounds is outlined in the Methods section
2. Protein (purified)
3. Pyrex culture tubes (Sigma catalog no. CLS98206)
4. 50 mM ammonium bicarbonate
5. 100 mM DTT
6. 500 mM iodoacetamide
7. 60% acetonitrile plus 0.2% TFA

8. 2.5% acetonitrile plus 0.2% TFA
9. 2.5% acetonitrile plus 0.1% formic acid
10. 97.5% acetonitrile plus 0.1% formic acid
11. 50% v/v acetonitrile plus 5% formic acid.
12. Water plus 0.1% formic acid
13. STAGE tip [24] containing C18 Empore Disks (3M)
14. Capillary column created by packing 5- μ m spherical C18 silica beads into a fused silica capillary (75- μ m inner diameter x 12-cm length) with a flame-drawn tip [25].
15. Trypsin (Promega, Madison, WI) or other suitable proteases

3.2. Equipment

16. Photoreactor- model UVL-56 20W Black-Ray handheld lamp (Upland) or Rayonet mini photochemical chamber reactor with RMR-3500 lamps.
17. Speed-vac (Thermo Scientific)
18. Gel electrophoresis apparatus
19. Mass spectrometer (LTQ linear ion-trap) or LTQ-FT or Orbitrap coupled with liquid chromatography and Software for mass spectral analysis, Xcalibur, SEQUEST and Biopharma Intact (Thermo Fisher)

4. Methods

4.1. Synthesis of azialcohols

Synthesis of 3-azibutanol [26], 3-azioctanol [27] and 7-azioctanol [19] is outlined in Figure 8. 3-keto butanol can be converted to the corresponding 3-azibutanol in the presence of hydroxylamine-*O*-sulfonic acid in liquid ammonia followed by pyridine/I₂. This synthetic step is also applied to the synthesis of 3-azioctanol and 7-azioctanol. For the synthesis of 3-azioctanol, first step involved the protection of the hydroxyl function of the starting material, 3-hydroxypropionitrile, with the acid-labile *tert*-butyldimethylsilyl group. The next step is the Grignard reaction of the protected nitrile with pentylmagnesium bromide followed by deprotection of the resulting ketone. The hydroxy ketone is then treated with hydroxylamine-*O*-sulfonic acid in liquid ammonia to give the corresponding diaziridine derivative which is subsequently oxidized to the diazirine by pyridine/I₂. The next step is the Grignard reaction followed by treatment with dilute HCl to have the corresponding 3-keto octanol. Finally treatment of 3-keto octanol with hydroxylamine-*O*-sulfonic acid in liquid ammonia and pyridine/I₂ gives 3-azioctanol. The first step of 7-azioctanol synthesis is by converting 3, 4-Dihydro-2-*H*pyran to 2-hydroxytetrahydropyran by HCl. Then, hydroxy-3-octen-7-one is synthesized from 2-hydroxytetrahydropyran by the Wittig reaction with phosphoniumtriphenyl-2-oxopropylide. Hydroxyoctan-7-one can then be converted to the corresponding 7-azioctanol by the reaction with hydroxylamine-*O*-sulfonic acid in liquid ammonia followed by pyridine/I₂.

4.2. Photoaffinity labeling

- a. A 10–15 μL aliquot of freshly purified protein is added to water or appropriate buffer to a final concentration of 2–4 μM protein in a total volume of 200 μL in a small pyrex culture tube (0.5 ml) purged with N_2/Ar gas. Appropriate amounts of aqueous 3-azioctanol, 7-azioctanol, or 3-azibutanol are preincubated for 30 min and the tube is sealed with parafilm.
- b. The tube is photoirradiated for 30 min using a model UVL-56 20W Black-Ray handheld lamp (Upland) or Rayonet mini photochemical chamber reactor with RMR-3500 lamps.

4.3. Mass spectrometry

4.3.1. Determination of stoichiometry of azialcohol photoincorporation—The following steps can be performed on the sample after the step b of section 4.2.

- a. A few microliters of acetic acid are added to the tube after photolabeling to a final concentration of 1%.
- b. The sample is directly infused into the mass spectrometer using a syringe pump. Mass spectra are acquired from m/z 300 to m/z 2000 with a maximum ejection time of 400 msec.
- c. Molecular weights for both the azialcohol-labeled and unlabeled forms of the protein are calculated from the resulting charge envelopes. Essentially, the charge state of an ion is determined by subtracting the mass to charge (m/z) ratio of two adjacent ions from each other and the reciprocal of this difference multiplied by the ion of lower mass/charge minus one. The result is the charge state of the ion with the higher m/z of the two adjacent peaks. To calculate the unprotonated molecular weight, the determined charge state is multiplied by the ion's mass/charge, and then the charge state is subtracted from the product. Mass values can be deconvoluted using Biopharma Intact module of Biopharma software from Thermo Fisher.

4.3.2. Site of photolabeling-Online reverse-phase chromatography and sequencing of azialcohol-labeled protein—The following steps can be performed on the sample after the step b of section 4.2.

- a. Photoincorporated protein (1 μg) is dried down by using a speed-vac and then resuspended in 45 μL of 50 mM ammonium bicarbonate.
- b. This solution is spiked with 5 μL of 100 mM DTT to a final concentration of 10 mM DTT and then heated at 62°C in a heating block for 30 min.
- c. 6 μL of 500 mM iodoacetamide (final concentration, 50 mM) is added and heating continued at 37°C for 20 min in the dark.
- d. The sample is dried under speed-vac and reconstituted in 2.5% acetonitrile plus 0.2% TFA solution.

- e. Each solubilized sample is loaded onto a STAGE tip [24] containing C18 Empore Disks (3M), eluted with an equal volume of 60% acetonitrile plus 0.2% TFA and dried in the speed-vac.
- f. Samples are reconstituted in 50 mM ammonium bicarbonate containing 12.5 ng/ μ L modified sequencing-grade trypsin (Promega) and placed in a 37°C room for no longer than 2 h.
- g. The reaction is stopped by completely drying the samples in a speed-vac.
- h. On the day of analysis, the sample is reconstituted in 5 mL of HPLC solvent (2.5% acetonitrile, 0.1% formic acid).
- i. A nanoscale reverse-phase HPLC capillary column is created by packing 5- μ m spherical C18 silica beads into a fused silica capillary (75- μ m inner diameter x 12-cm length) with a flame-drawn tip [25].
- j. After equilibrating the column with water containing 0.1% formic acid, each sample is pressure-loaded offline onto the column. The column is reattached to the HPLC system. A gradient is formed and peptides are eluted with increasing proportions of solvent (97.5% acetonitrile, 0.1% formic acid).
- k. As each peptide is eluted, it is subjected to electrospray ionization and then entered an LTQ linear ion-trap mass spectrometer (Thermo Fisher). Eluting peptides are detected, isolated, and fragmented to produce a tandem mass spectrum of specific fragment ions for each peptide. Mass values of 52 Da for an iodoacetamide-modified cysteine residue, 72 Da for 3-azibutanol and 128 Da for 3- and 7-azioctanol are added to the mass value of the parent peptide for looking into these modifications. In some cases, for greater confidence in peptide identification, high mass accuracy can be obtained by analyzing samples on an LTQ-FT mass spectrometer (Thermo Fisher).
- l. Peptide sequences are determined by matching the peptide library generated by the used protease against the protein of interest or the protein databases with the acquired fragmentation pattern by the software program SEQUEST (Thermo Fisher) [28].

4.3.2. In Gel digestion—The following steps can be performed on the sample after the step b of section 4.2.

- a. Approximately 10 μ g of photolabeled protein is separated by SDS-polyacrylamide gel electrophoresis using a 10% polyacrylamide gel followed by Coomassie blue staining.
- b. The band corresponding to the molecular weight of the protein is cut into ~2 mm³ pieces and incubated overnight at room temperature with 200 μ L of 50 mM ammonium bicarbonate solution, and then washed with acetonitrile for 10 min. After the gel bands are cut up, the coomassie stain was removed by adding a solution of 50% acetonitrile/50% 50 mM ammonium bicarbonate. The solution sits until solution turns blue, which usually takes a few minutes. If the gel is

heavily stained it takes several exchanges of the liquid. After this step the gel is shrunk down with acetonitrile. The acetonitrile is removed and the gel is then dried in a speed-vac and is ready for the enzyme to be added or for reduction/alkylation steps.

- c. 1M DTT (in 50 mM ammonium bicarbonate) is added to sufficiently cover the gel and account for swelling of the gel. The tube is marked when the gel is fully hydrated prior to shrinking down the gel with acetonitrile to make it easier to add enough volume. The tube is then incubated at 60 °C for 30 minutes. Then the liquid is removed and the gel is shrunk with acetonitrile. Acetonitrile is then removed, the gel is dried and 5M iodoacetamide (in 50 mM ammonium bicarbonate) is added, enough to account for the swelling of the gel. The reaction is done at room temperature in the dark for 15 minutes. The solution is then removed and acetonitrile is added to shrink the gel. The acetonitrile is removed and the gel is dried. The gel is now ready for protease digestion.
- d. The gel pieces are rehydrated with aqueous 50 mM ammonium bicarbonate solution containing 12.5 ng/μL modified sequencing-grade trypsin at 4°C.
- e. After 45 min, the excess trypsin solution is removed and replaced with a volume of 50 mM ammonium bicarbonate solution that just covered the gel pieces, which are then incubated at 37°C overnight.
- f. Peptides are extracted from the gel pieces by first removing the ammonium bicarbonate solution and then performing two consecutive 20 min incubations in a solution composed of 50% v/v acetonitrile and 5% v/v formic acid.
- g. The extracts are combined, dried under vacuum for ~1 h and stored at 4°C until analysis.
- h. Sample is analyzed as described for steps j-l in section 4.3.2.

5. Notes:

- a. The concentrations of the azialcohol can be calculated using the molar extinction coefficient 3-azibutanol as 55 at 347 nm [26] and 56 at 350 nm for 3-azioctanol or 7-azioctanol.
- b. The photoreaction can also be performed using quartz cuvette. The reaction time can be determined by measuring the half-life of the azialcohol under the same condition. The half-life is measured by monitoring the decrease in absorbance at 350 nm with time upon photoirradiation.
- c. The protein and the azialcohol ratio should be selected carefully. Appearance of cloudiness in the solution after azialcohol addition indicates the precipitation of protein. In that case, concentrations of azialcohol should be reduced.
- d. Photolabeling should be conducted under inert atmosphere. In the presence of oxygen, additional photoproducts can be generated. Oxidation of methionine has

been detected in the mass spectrometric analysis of photolabeled PKC δ C1B [12].

- e. Sometimes bubbles are seen in the reaction mixture after photoirradiation in the sealed tube indicating the release of nitrogen gas from the photoreaction.
- f. Buffer composition can affect photolabeling efficiency. For example, photolabeling efficiency of 3-azidoctanol with PKC δ C1B is higher in Hepes buffer than in Tris buffer [29].
- g. Photolabeling is frequently associated with non-specific labeling. In order to rule out nonspecific labeling, photolabeling can be performed using varying concentrations of azialcohol. Photolabeled residue(s) at lowest concentration of the azialcohol site(s) of highest affinity. The specificity of the alcohol binding site can also be measured by conducting the photolabeling experiment in the presence of ethanol/butanol/octanol. The presence of these parent drugs will block the azialcohol at the binding site resulting in reduced photoincorporation. The extent of photoincorporation can be accurately measured using radiolabeled azialcohols [17, 19, 27].
- h. To understand the biological significance of the photolabeled residue(s), they should be mutated and activity/binding of the mutant proteins for alcohol should be measured.
- i. Appropriate protease or protease combination should be used for protein digestion to obtain highest amino acid coverage of a protein.
- j. 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.
- k. Quantitation of the photolabeling residues can be done using heavy isotope labeling methods such as, Stable Isotope Labeling of Amino acids in Cell culture (SILAC) and Tandem-Mass-Tag labeling and/or spectral counting methods [30, 31].
- l. Quantitation of the photolabeled residues are usually done using radioactive methods. Photolabeling is conducted using radiolabeled azialcohol, followed by protease digestion and Edman sequencing [17].

6. Conclusion:

Photoaffinity labeling combined with mass spectrometry is a valuable tool in identifying alcohol-binding residues in proteins. With the rapid advancement of high resolution mass spectrometers it is possible to identify photolabeled peptides and the corresponding amino acids unequivocally. The methodology described here can also be applied to the diazirine analogs of other drugs with a few tweaks here and there. It is important, however, to consider a few points while interpreting the photoaffinity labeling and mass spectrometry data, which include, a) attaching a diazirine moiety to the chemical structure of a drug may alter its binding properties, b) generated carbene may have reactivity preferences for certain amino acids, and c) values of parameters such as, Xcorr and dCn, should be in the optimal range while identifying photolabeled peptides and amino acids during mass spectral data analysis using the SEQUEST algorithm.

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3-azibutanol

3-azioctanol

7-azioctanol

aziethanol

Figure 1:
Chemical structure of azialcohols.

Figure 2:

Possible degradation pathways of aziethanol. The hydroxyl group oxygen can attack the carbon atom of the diazine ring and form an epoxide. In the presence of water molecule the epoxide can be opened up to form ethylene glycol.

Figure 3:
Photoconversion of diazirine compounds to diazo and carbene. R and R' represent alkyl or aryl groups.

Figure 4:

Simplified scheme for the detection of azialcohol photoincorporation into proteins. In the mass spectrometric method, the photolabeled protein can be analyzed either by MALDI-TOF or by ESI mass spectrometer to identify photoincorporation and stoichiometry. The sites of photoincorporation are detected by acquiring MS/MS data for the digested sample in ESI mass spectrometer.

Figure 5:

Stoichiometry of photoincorporation of 3-azibutanol (1 mM) into Munc13-1 C1. Molecular weight of the purified Munc13-1 C1 is 6754 Da. The peaks at 6826 Da, 6898 Da and 6970 Da represent photoincorporation of 1, 2 and 3 molecules of 3-azibutanol respectively. Addition of one molecule of 3-azibutanol causes an increase of 72 Da. Inset: the charge envelop of the photolabeled Munc13-1 C1 obtained after the sample was infused into the LTQ linear ion trap mass spectrometer.

Figure 6:

Identification of the photolabeled residues in Munc13-1 C1 by LC/MS/MS after tryptic digest. Glu-582 as the site for 3-azibutanol: MS/MS data for the 3-azibutanol (0.1 mM) modified 16-mer peptide TATTPTYCYECEGLLW. In addition to the singly charged ions, double charged b_{13}^{++} , b_{14}^{++} , and b_{15}^{++} are also observed. 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 72 Da for 3-azibutanol added to them. Observed values are shown in bold.

Figure 7: Alcohol binding site in Munc13-1 C1 determined using photolabeling and mass spectrometry. Photolabeled residue Glu-582 is shown in magenta in both ribbon (left) and surface (right) diagrams. The models are generated using the solution structure of Munc13-1 C1 (PDB ID: 1Y8F) and visualized using the UCSF Chimera package from the Computer Graphics Laboratory, University of California San Francisco, San Francisco, CA, U.S.A.

A **B** **C**

Figure 8:
Synthetic scheme for A) 3-azibutanol, B) 3-azioctanol and C) 7-azioctanol.