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COMMENTARY

CHEMICAL MECHANISMS FOR PHOTOAFFINITY LABELING OF RECEPTORS

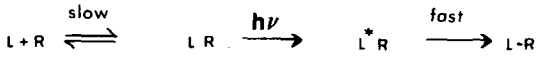
DAVID CAVALLA* and NORTON H. NEFF†

Laboratory of Preclinical Pharmacology, National Institute of Mental Health, Saint Elizabeths Hospital, Washington, DC 20032, U.S.A.

A recent commentary in *Biochemical Pharmacology* [1] focussed on the use of photoaffinity labels as tools in organ bath experiments, and also gave an exhaustive review of nearly every application of this technique in the labeling of receptor proteins. This paper will concentrate on the theoretical and chemical basis for photoaffinity labeling in the hope of imparting a clear understanding of the pitfalls and reasons for the successes of the method, and identify ways in which improvements may be made.

The ideal case

Photoaffinity labeling has achieved prominence in the field of biochemical pharmacology because it offers a relatively specific method of "tagging" a ligand-binding molecule [2]. In the sense that it forms a covalent bond between a binding site and its ligand, it has similarities to affinity labeling [3, 4] and cross-linking [5–7]. However, with photoaffinity labeling, the functional group responsible for the labeling is attached to the ligand in a latent form. A major advantage of the technique is that the ligand may be photoactivated (by ultraviolet or short-wave visible light) at a time of the experimenter's choosing. Prior to irradiation, an equilibrium is established between ligand and binding site which sets up these two components in close proximity and, therefore, well situated for covalent bond formation. In the ideal case, photoactivation of the ligand produces a reactive intermediate which reacts instantaneously with its binding site (Scheme I). Unbound ligand is photoactivated and reacts with its solvent cage or other



Scheme I

close environment. Thus, in the ideal case, irradiation "freezes" the reversible association between photolabile ligand and binding site into an irreversible labeling of the site by photodecomposed ligand. Non-specific binding is also frozen, and the specific to non-specific ratio of irreversible binding mirrors that ratio for reversible binding.

* Address all correspondence to: Dr. David Cavalla, Glaxo Group Research, Chemistry Department, Ware, Herts., England SG12 0DJ.

† Present address: Department of Pharmacology, The Ohio State College of Medicine, 5086 Graves Hall, 333 West Tenth Ave., Columbus, OH 43210.

Potential of photoaffinity labeling

Nearly every known neurotransmitter receptor has been photolabeled (for a review, see Ref. 1), but although this is often interesting work, the technique has greater potential. For instance, the goal of receptor isolation, an ambitious one in biochemical pharmacology, has made good use of photoaffinity labeling. The isolation generally requires solubilization of the receptor from a membrane preparation, followed by successive chromatographic purifications: size exclusions, ion exchange and, most important, affinity chromatography [8, 9] are performed sequentially. At a certain point, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is required to assay the purity of the preparation. Coomassie blue staining of the gel indicates how many proteins are present, but autoradiography of a photoaffinity-labeled preparation indicates which one(s) contains the binding site for the ligand. As an example, the recent isolation of the β-adrenergic receptor and purification to homogeneity represent significant achievements [10, 11]. Its reconstitution with purified guanine nucleotide regulatory protein (N_s) has been shown to confer hormone responsiveness on the resolved catalytic unit [10, 12]. A highly radioactive specific photoaffinity label (*p*-azido-*m*-[^{125}I]iodobenzylcarazolol) [13, 14] has been integral to the success of the isolation, which requires a purification of about 100,000-fold, and is orders of magnitude more difficult than purification of the nicotinic acetylcholine receptor from *Torpedo*.

Some information about the activation and desensitization of the acetylcholine receptor has been revealed recently by a photoaffinity probe of the ion channel, chlorpromazine. This compound is a noncompetitive blocker of acetylcholine receptor function and is believed to bind to a site within the channel. When irradiated, [^{3}H]chlorpromazine was bound irreversibly, a process that was dose-dependently facilitated by short-term incubation with acetylcholine and other agonists, blocked by tubocurarine, and desensitized by long-term incubation with acetylcholine [15]. A similar experiment was performed with quinacrine azide as the label [16]. Because acetylcholine receptor desensitization is so rapid, the photolabeling times were very fast (< 20 msec), and required stopped-flow techniques. These experiments are the biochemical correlates of electrophysiological measurements, in which the effects of agonist-induced activation, antagonist

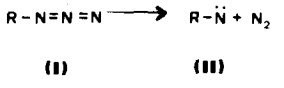
blockade and agonist-induced desensitization on ion flow are observed.

In addition to revealing basic information on how receptors work, the photolabeling technique has been used to identify the difference between the binding sites for the lactogenic and somatogenic hormone receptors. In combination with antibodies specific for each receptor, the binding subunits were shown to have different relative molecular weights [17]. The study clarified some conflicting reports concerning the nature of the binding sites for human growth hormone in rabbit liver.

These various examples are testimony to the power of photoaffinity labeling as a biochemical technique and how it may succeed where reversible binding fails in receptor characterization. However, there is room for improvement: sometimes not enough protein is radiolabeled for its easy detection on a gel because of the low *efficiency* of a photolabeling process; sometimes the protein may lose its label through subsequent handling or preparation for SDS-PAGE because the photolabeling is not *stable*; and sometimes we would like the labeling to be *specific* for one amino acid residue in the binding site. This would allow the label to be used as a probe for the primary structure of the binding site. The meaning of these italicized terms, and the reasons for the problems still to be resolved, are now enlarged upon.

Use of aryl nitrenes

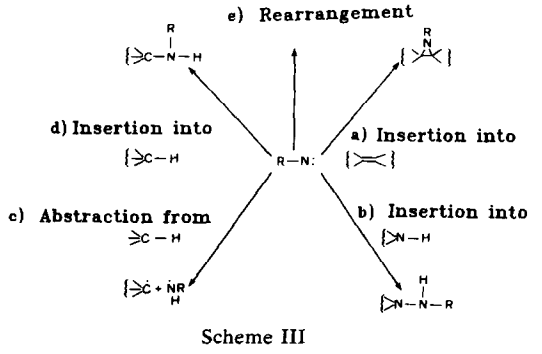
Nitrenes (II) are extremely unstable valence-deficient derivatives of nitrogen that are produced from azides (I) through loss of a molecule of nitrogen (Scheme II). A favorable free-energy change for the



Scheme II

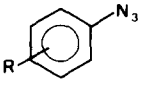
reaction is guaranteed by the stable nature of the N_2 molecule, and mild doses of u.v. irradiation are sufficient to drive the reaction forward. Indeed, the predictability of azide photodecomposition is a major determinant in the popularity of these compounds as photolabeling agents.

Because of the extreme unstability of these nitrenes, they show a great deal of diversity in their reactions (Scheme III). While this may be an advantage if the binding site is barren of a functional group that will react with an alternative labeling agent, it is also a disadvantage: only some of the reactions of nitrenes will produce a ligand-binding site covalent bond (routes a, b and d, Scheme III). Rearrangement is one of the reactions that does not result in photolabeling. With some substituents, R, this pathway is predominant—thus alkyl nitrenes are disbarred. Acyl nitrenes (R = R'CO), though less susceptible, will usually undergo the Curtius rearrangement to an isocyanate. For details on the chemistry of these processes, the reader is referred to Refs. 2 and 18. These considerations explain the low efficiency of the photolabeling process. Efficiency is defined as the probability of a reversibly bound ligand becoming an irreversibly bound one. Aryl-substituted nitrenes (R = aryl), in addition to being good u.v. absorbers, are the least susceptible to rearrangement and give the highest efficiencies, though on average this is still only 10%.

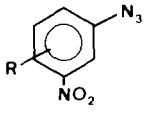


Scheme III

Despite such a figure, aryl nitrenes have been routinely used as photolabile groups. Large peptides may often be modified simply by appendaging a group such as (III) or (IV) without significantly



(III)

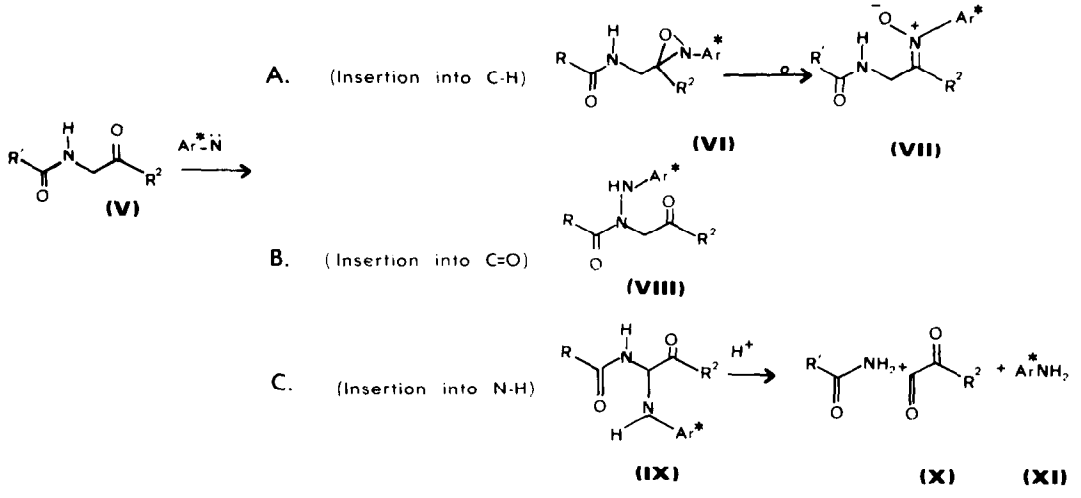


(IV)

impairing the ability of the peptide to bind to its receptor. This method has been successfully applied to the photoaffinity labeling of a number of peptide hormone receptors [19–25]. Smaller ligands may be suitably substituted around their aromatic nucleus by an azide group. This functional group is fairly small and may often be accommodated without diminishing receptor affinity. Examples of such modified ligands cover the great majority of neurotransmitter receptors and are too numerous to list here; the reader is referred to some recent reviews [1–3]. Despite the abundant success, the low labeling efficiency can still be an obstacle: in tissues that contain especially low concentrations of binding sites the quantity of radioactivity incorporated may be too small to be easily detected. This problem is most severe for tritium and ^{14}C-radiolabeled proteins, while ^{125}I-labeling (such as is usually possible with peptides) confers sufficient specific activity to be nearly always detectable.

Stability

Dogma would dictate that, by establishing a covalent bond, the labeling becomes irreversible. The problem of how stable that "irreversible" labeling is has rarely been addressed, since it is very difficult to answer quantitatively: kinetically, the time course of decomposition of a covalent complex between a ligand and its binding site may appear similar to the dissociation of a reversibly bound complex. Nevertheless, a cursory chemical analysis [26] would indicate stability to be a problem that would lower the effective efficiency even further. In terms of the goals of photoaffinity labeling, the bond should be stable

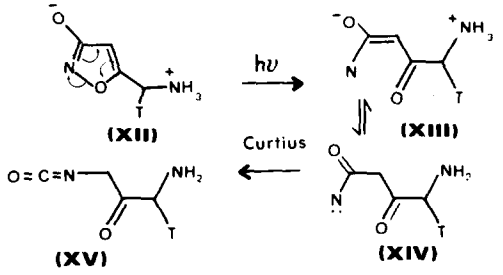


Scheme IV

to the procedures involved up to and including the visualization of the protein by autoradiography. Such conditions include u.v. light, acid (for gel-fixing), heat and β-mercaptoethanol (gel sample preparation). Stability with respect to other conditions may be important for other experiments, and these should be considered individually by the experimenter.

Scheme IV shows a few of the structures one would expect from the reaction of an aryl nitrene with a peptide chain. Route A considers insertion of a nitrene into a carbonyl group. The first formed oxaziridine (VI) has the capacity to rearrange, a reaction facilitated by ultraviolet light, to give the nitrone (VII) which may easily hydrolyze and lose the radiolabeled aryl group (Ar*). Route B gives a hydrazine (VIII) which, for the purposes of the experiment, may be considered quite robust. Route C gives an amino-amido acetal (IX) which may be hydrolyzed and split in three to give an amide and α-ketoaldehyde (X). The radioactive portion of the photolabel is lost as an amine (XI). The chance of hydrolysis occurring during washing of the preparation to remove reversible binding is slight. However, acidic conditions (such as used in gel-fixation) or heat (such as in the preparation of samples for loading onto a gel) may well be severe enough to cleave the radioactive label from the protein. This analysis, though entirely theoretical, lends credence to the idea that not all photolabeling is irreversible. Indeed quite mild conditions may break the photolabeling bond. In addition to the reactions between a nitrene and protein considered here, for a generalized protein chain, there are many diverse others with any of the side chains of the various amino acids. In general, insertion into a CH bond of a CH_2 group flanked by two other CH_2 groups will yield a bond stable to all further manipulations. On the other hand, insertion into a C-H bond next to a serine hydroxyl group gives an aminal, which may be cleaved by acids and the radiolabel lost.

A different kind of instability is probably involved in the photolabeling of the γ-aminobutyric acid (GABA) receptor by [^{3}H]muscimol [27]. The GABA receptor is difficult to photolabel by an aryl nitrene, since no convenient modification of an aromatic agonist by an azide group exists. However, [^{3}H] muscimol (XII) itself produces an aryl nitrene (XIV) on photolysis (Scheme V). Though Curtius



Scheme V

rearrangement can give an isocyanate (XV), the degree to which this interferes with the photolabeling is unclear; such a product may itself act as an affinity label for the binding site. In any case, the photodecomposition produces a ketone group adjacent to the tritium label in XIV, and this structure will be maintained in the photolabeled protein. Through generation of the ketone group, the tritium, now in an enolisable position, is readily exchanged with water. Though protected to a certain extent by a β-carbonyl on the other side of the ketone, it is apparent that some radiolabel is lost during acidic gel fixation and preparation for autoradiography [27]. Such instability may contribute to the difficulty some workers have experienced in repeating the original procedure [28]. In addition to chemical instability, the radiolabeled protein is also subject to proteolysis; unless precautions are taken, a single photolabeled receptor may be decomposed to multiple forms by endogenous proteases [29, 30].

The degree to which the chemical forms of decomposition lower the overall labeling efficiency is difficult to ascertain. In general, a nitrene will produce a number of different covalent bonds, some stable, some not, and their relative proportion reflects the

proximity of the nitrene to each group. The spatial orientation will also determine the proportion of non-productive reactions (e.g. rearrangement, H-abstraction) versus productive reactions (e.g. insertion). The type of bond formed also depends on the relative proportion of nitrenes in the singlet and triplet states: for example, the singlet state shows a greater preference for insertion into O—H and N—H over C—H bonds [2]. Thus, the efficiency of the labeling process may vary from 0 to 20%, when using the same ligand to label different proteins [31].

Specificity

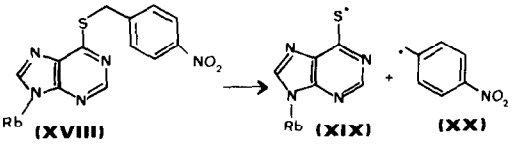
Unfortunately, some of these problems seem to derive *a priori* from our "ideal case," in which the photodecomposed ligand reacts immediately with its surroundings. Immediate reaction implies high reactivity, and high reactivity implies non-specificity. In this sense, non-specificity means that a number of amino acid residues are labeled around the binding site. One ideal of photoaffinity labeling is its use in probing the binding site: extensive proteolysis of a photolabeled receptor protein to generate a large number of small peptides would theoretically produce only one containing radioactivity. The sequence of this peptide would tell the primary structure of (part of) the binding site. In reality, because a number of amino acid residues are often labeled, such an experiment is very difficult, and very rarely performed successfully (only once, to the authors' knowledge, Ref. 32).

To solve the problems of efficiency, stability and specificity is not easy: in many respects aryl nitrenes are the best available. One of their main advantages is predictability: one needs to know nothing about photochemistry except that aryl azides always photodecompose to aryl nitrenes. Nevertheless, there have been improvements on aryl nitrene photolabeling, but the examples are more diverse in their chemistry and less widespread in their application.

Reversible photoactivation

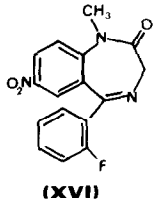
These examples may be grouped under the term reversible photoactivation because the photoactivated ligands may revert to the ground state if they do not react. The photoactivated state often represents an electronically excited state of the molecule. Unlike aryl azides, these ligands have more than one opportunity to produce a covalent bond. So long as they remain in the binding site they may be photoactivated numerous times.

As a result, with the correct chemistry, high labeling efficiencies may be achieved. One major success has been the photolabeling of the benzodiazepine recognition site with flunitrazepam (XVI). As assessed by the ability of this compound to permanently impair reversible binding capacity, nearly stoichiometric labeling efficiency is achieved [33]; however, as assessed by the amount of radioactivity irreversibly incorporated, the efficiency is around 25%. The underlying basis for this discrepancy is still unclear. In addition to providing a molecular weight for the binding protein, the method has produced an *in vitro* assay for the distinction of agonist and antagonist classes of ligands [34]. As another example, the nitrostilbene (XVII) gave a 51% efficiency of labeling of the estrogen binding site [35]. Nitrobenzylthioinosine (XVIII) gave a 50% yield of labeling of the adenosine uptake site [36–40]. Although all these compounds possess one structural feature in common (the nitro group), beyond enhancing the u.v. absorption of the ligand, this is not the major factor involved in the photolabeling: in fact, each example expresses a different photochemistry which may have more general applicability. Flunitrazepam (XVI) probably cleaves at the amide nitrogen bond to give a diradical which combines with a suitable functional group at the binding site [41]. Tyrosine has been proposed as a

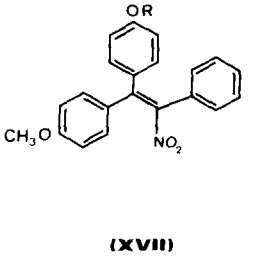


Scheme VI

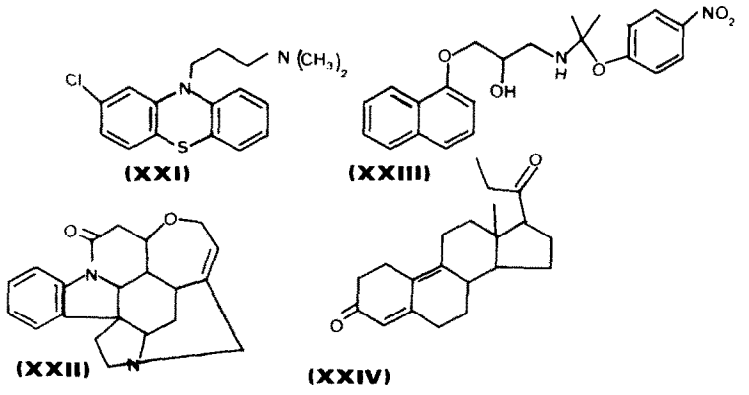
candidate for this reaction. The nitrostilbene (XVII) is chemically similar to a nitroanisole; such compounds are known to undergo photostimulated substitution by hydroxide ion, primary amines, etc. [42]. By analogy, a serine, tyrosine or lysine residue could conceivably be responsible for the photolabeling bond in this case. Nitrobenzylthioinosine (XVIII) probably splits into two radicals, (XIX) and (XX), either one (or both) of which might bond covalently. It is interesting to speculate whether if, in this case, both of the halves of the molecule were radiolabeled, the labeling efficiency would be higher than it is (50%). These examples are by no means unique. Cleavage of an acyl-sulfur bond is probably implicated in the photolabeling of the acetylcholine receptor channel by the non-competitive blocker chlorpromazine (XXI) [43]. The efficiency is lower than for nitrobenzylthioinosine (XVIII), reflecting the greater stability of the benzyl over the phenyl radical. Strychnine (XXII) has been used as a photoaffinity label of the glycine receptor [44]; cleavage of the amide bond (similar to flunitrazepam) is the best candidate for the mechanism of this reaction. The lack of a nitro group does not seem to greatly impair the labeling efficiency, which is 35%. Nitroanisoles have also found use in the photoaffinity labeling of the β-adrenergic receptor: the propranolol derivative (XXIII) elicited a 58% labeling efficiency, as judged by loss of [^{3}H]dihydroalprenolol binding sites [45]. Finally, in a different kind of application, the nitro group on the Ca^{2+} channel blocker, nifedipine, enabled the photostimulated conversion of this compound into an inactive sub-



(XVI)



(XVII)



stituted pyridine, and was used to show that Ca^{2+} entry directly activates contraction in the frog heart [46].

The carbonyl group displays a wide array of photochemical reactions, particularly when conjugated with a double bond—as an enone or dienone. Indeed, compounds containing such functionality have been proposed as photoaffinity reagents for Δ^5-ketosteroid isomerase [47]. The steroid (XXIV) has been used as a probe for the progesterone receptor [48, 49] and eventually gave a 50% labeling efficiency. Limited proteolysis of the photolabeled receptor established structural homologies in the two binding sites from different subunits of this protein [50].

There are other examples where underivatized ligands have been used in photoaffinity labeling. Most give very low efficiencies, although the simplicity of avoiding chemical modification may confer certain advantages. In this list are included the labeling by dopamine of its receptor [51, 52] or uptake site [53], by trimethisoquin [42] and phencyclidine [42] of the acetylcholine receptor channel and by nitrendipine of the Ca^{2+} channel [30]. The peptides bungarotoxin [54] and cholecystokinin-33 [55] have also been used in underivatized form to label their respective binding sites. The wide range of structures involved here makes many hitherto uninvestigated compounds candidates for photoaffinity labeling. However, the greatest successes in non-nitrene photolabeling have come from a fairly limited range of structures. These are nitroaryl compounds further substituted with amide, ether or thio groups (e.g. XVI, XVII and XVIII respectively).

In all these examples, the radicals produced from photoactivation are less reactive than nitrenes. The chance of them producing a permanent covalent bond may be small, but repeated photoactivation multiplies the yield. With regard to long irradiation times, the limiting factor may only be the destruction of the binding site by u.v. light. For Δ^5-ketosteroid isomerase, photolabeling times were sometimes extended up to 24 hr [47]. Second, because of their lower reactivity, reversibly photoactivatable ligands are likely to be more specific in their reactions. For instance, with nitroaryl ethers the labeling amounts to a photoactivated nucleophilic substitution. In this case, a correctly oriented nucleophilic group will be required for the coupling. Third, the bonds to be expected from the photolabeling by compounds considered here will be relatively stable to acid and heat.

In conclusion, photoaffinity labeling is now a powerful technique for the biochemical investigation of receptor and their action. In this respect the use of aryl nitrenes has been very successful, but there are limitations imposed by the chemistry of the labeling process. The predictable photochemistry of aryl azides, and the highly reactive nature of the nitrenes so generated have made the procedure popular, but in most cases the labeling efficiency is low, the labeling bond sometimes unstable, and the specificity with respect to amino acids within the binding site poor. To extend the frontiers of photoaffinity labeling, different photochemical reactions, which can improve these aspects, need to be investigated. To this end, nitroaryl substituted ethers, amides and thiols are three groups of compounds which are particularly worth investigating.

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