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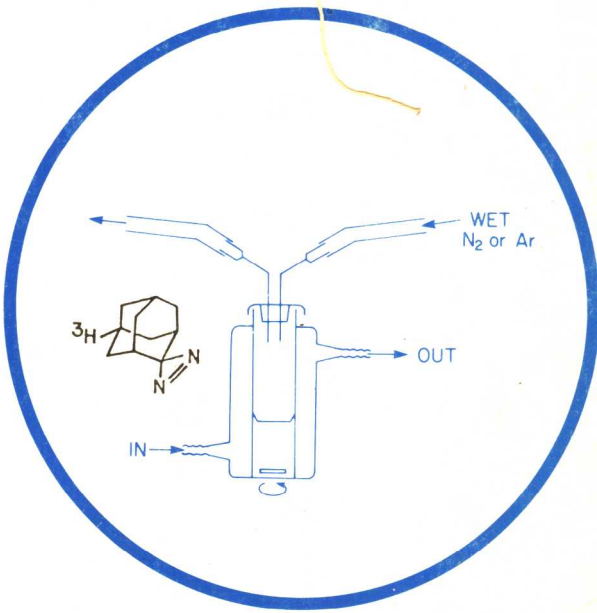

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PHOTOGENERATED REAGENTS IN BIOCHEMISTRY AND MOLECULAR BIOLOGY

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Preface

Fortunately, the experiments that can be done with photochemical reagents are too varied (and unpredictable) to allow the writing of a true laboratory manual. Instead I have tried to give an account of the possible experiments (Chapter 1), a description of the reagents that can be used (Chapter 2), and a discussion, rather than detailed protocols or dogmatic assertions, of how the experiments can be done (Chapters 3 to 6). In addition, the extensive bibliography of over 400 references will provide access to useful examples in the primary literature.

This monograph is an expansion and revision of the review I wrote earlier with my mentor Jeremy Knowles (Bayley and Knowles, 1977). Jeremy's earlier short review remains an excellent summary of the essential idea of photoaffinity labeling. Another review that will remain valuable is from Frank Westheimer's laboratory where photoaffinity labeling was invented (Chowdhry and Westheimer, 1977). Westheimer has also written a delightful short history and prospectus of the subject (Westheimer, 1980). Several additional reviews concerning special aspects of photochemical reagents are cited in this text.

I would like to thank my friends and colleagues who read the first draft, gave me many ideas for amendments, and told me of omissions and indeed a few errors. They were Josef Brunner, Mike Dockter, Boyd Haley, Arthur Karlin, John Katzenellenbogen, Koji Nakanishi, Fred Richards, Jim Staros and Bernadine Wisnieski. I also thank the many who sent preprints and reprints. I am indebted to the editors for their assistance and forbearance, particularly over the delay caused by my move to Columbia, to Marjorie Dunn who typed the first draft, to Olga Hanlon who produced the final version, and to all the others who helped me put together the manuscript.

I hope the reader will inform me of errors, omissions, or differences of opinion. I do regret not including a full chapter on crosslinking experiments with nucleic acids, and perhaps that can be repaired in the future.

HAGAN BAYLEY
Manhattan, New York, 1983

List of abbreviations

ACTH	corticotropin
AMP, ADP, ATP	adenosine 5′-mono-, di-, and triphosphates
atm	atmosphere (pressure)
cAMP, cGMP etc.	cyclic adenosine 3′:5′-monophosphate, etc.
CF_1	coupling factor 1, the hydrophilic portion of the proton-translocating ATPase of chloroplasts
CoA	coenzyme A
DEAE	diethylaminoethyl
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dpm	decays per minute
DTT	dithiothreitol
EFG	elongation factor G
ϵ	molar extinction coefficient
Fab	monovalent immunoglobulin G fragment
GMP, GDP, GTP	guanosine 5′-mono-, di-, and triphosphates
HPLC	high pressure liquid chromotography
IEF	isoelectric focusing
IgG	immunoglobulin G
IR	infrared
K_d	dissociation constant
K_i	inhibition constant
K_m	Michaelis–Menten constant
λ_{max}	absorption maximum

Na,K-ATPase	sodium and potassium activated adenosine triphosphatase (Na pump)
NAD	nicotinamide adenosine dinucleotide
NEM	*N*-ethylmaleimide
N_3XMP etc.	Azidopurine nucleoside phosphates (8-azido derivatives unless otherwise indicated)
pUp	3′:5′-uridine diphosphate
ψ	quantum yield
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
THF	tetrahydrofluorine
TLC	thin layer chromatography
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
UV	ultraviolet

Contents

The utility of photoaffinity labeling and related methods

Many biological molecules may be classified as ligands; others may be classified as receptors. For the purpose of this monograph it has proved convenient to extend the common usage of these terms (cf. Knowles, 1972). Here, ligands include enzyme substrates, allosteric effectors, haptens, neurotransmitters and hormones, and they bind to receptors, a term which embraces enzymes, immunoglobulins and the molecules which bind hormones, neurotransmitters and the like. Receptors are generally proteins. Ligands are more varied in structure and among them we find amino acids, sugars, nucleic acids, oligomers of these, the varied products of cellular metabolism, and foreign substances such as drugs.

Affinity labeling has been developed for investigating ligand–receptor interactions. The usual goal is either to identify a receptor in a mixture of candidates, which might be the polypeptides associated with a biological membrane or the subunits of a purified protein, or to locate one or more of the amino acid residues that make up the binding site of a receptor. In a chemical affinity labeling experiment (Singer, 1967; Shaw, 1970a,b; Glazer et al., 1975; Jakoby and Wilchek, 1977) the receptor is incubated with a modified ligand containing a functional group that, it is hoped, will react covalently with a residue at the binding site (Fig. 1.1). For instance, haloketones react with nucleophiles and one of the first successes of the affinity labeling technique was the identification of a histidine residue at the active site of chymotrypsin with *N*-tosyl-L-phenylalanine chloromethylketone (Schoellman and Shaw, 1963).

A useful chemical affinity reagent reacts more rapidly within the binding site than elsewhere because of its high 'local concentration' and, in the case of enzymes, because nucleophiles at the active site may be unusually

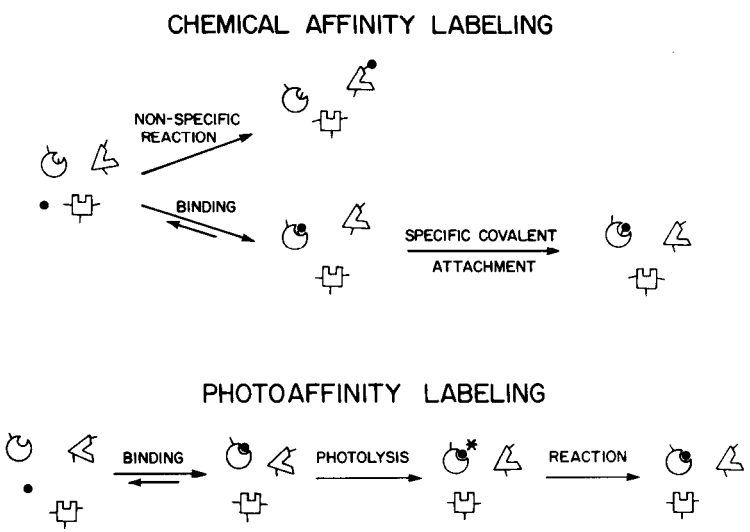

Fig. 1.1. Chemical affinity labeling and photoaffinity labeling. In a chemical affinity labeling experiment (*upper*) the ligand contains a reactive group designed to react with a functional group that might be present at the binding site of the receptor that recognizes the ligand. Under unfavorable conditions reaction may occur between the ligand and groups outside the binding site on the same protein or on a different molecule. For example, there might not be a suitably reactive or correctly oriented group at the binding site. In a photoaffinity labeling experiment *(lower)*, the ligand contains a photoactivatable group. Reaction can be initiated after binding is complete, eliminating one source of non-specific labeling, and a particular functional group need not be present at the binding site as the most reactive photogenerated species can react even with carbon–hydrogen bonds. This is not to say that complications cannot arise (see Chapters 3 and 4).

reactive. The use of radiolabeled reagents greatly facilitates the identification of intact receptor subunits and derivatized fragments of them.

1.1. The advantages of photogenerated reagents

A photoaffinity reagent (Fig. 1.1.) is a ligand that is chemically inert but conceals a highly reactive intermediate that is unmasked by irradiation with

near ultraviolet or visible radiation. The goals of photoaffinity labeling experiments are the same as those sought by chemical affinity labeling but the photochemical approach has several considerable advantages.

First, an inert ligand is desirable because it allows the investigator to perform important preliminary experiments with ease. Binding measurements and assays of biological activity are straightforward and unusual experiments that might not be feasible with a chemically reactive molecule can be done. For example, photolabile molecules may be used as haptens for antibody production and the hapten–immunoglobulin interaction subsequently explored (Fleet et al., 1969).

Second, the reaction of a photoaffinity reagent is initiated at will. In the case of kinetically inaccessible receptors such as those within living cells this is of crucial importance as chemical reagents may react with components of a biological preparation in a potentially misleading manner before they reach their target. The binding step has even been performed in living animals, and subsequently tissues have been removed and irradiated (see Section 4.2.). In favorable cases unbound photoaffinity reagents may be removed after the appropriate incubation period and the receptor–ligand complex rapidly irradiated before dissociation occurs, further reducing non-specific labeling. In areas related to photoaffinity labeling, bifunctional reagents containing both a chemically reactive group and a photochemically activatable group have been used in two-stage crosslinking experiments (see Chapter 5) and photochemical surface labeling reagents for membranes have been trapped inside cells before activation (see Chapter 6). In short, it is, in principle, less difficult to confine the reaction of a photogenerated reagent to the desired site.

A further advantage of photoaffinity reagents that reinforces the last conclusion is that intermediates formed by photolysis (see Chapter 2) are usually far more reactive than chemical reagents. Indeed, most of them are so reactive that it would be impossible to synthesize them and then add them to a receptor preparation. The most reactive photogenerated species can even attack functional groups such as carbon–hydrogen bonds which remain inert towards chemical reagents. In contrast, several different functional group-specific reagents may have to be tested before one suited to a particular problem is found. Extreme pH values, high temperatures or

pre-reduction of disulfide bonds may be required for efficient reaction of a chemical reagent, and hydrophobic binding sites, largely containing hydrocarbon residues may prove to be impossible to derivatize. Misleading results can be obtained when attempts are made to label an inert binding site with a chemical reagent. The ideal photoaffinity reagent will not misbehave in this way.

Finally, these properties permit the use of photoaffinity reagents in experiments in which kinetic phenomena are examined, and it has proved possible to extend the resolution to the millisecond time-scale.

1.2. Some examples of photoaffinity labeling and related experiments

To induce the reader to proceed further, the remainder of this short chapter gives a brief account of some applications of photogenerated reagents. Five classes of photoaffinity labeling experiments are described followed by three examples of the related methods that are discussed in the later chapters of this book.

1.2.1. The identification of a receptor in a mixture of proteins

The insulin receptor is present in trace amounts in the plasma membrane of liver cells. Using radiolabeled arylazido derivatives of insulin, which on irradiation yield reactive nitrenes, components of the receptor with molecular weights of 135,000 and 90,000 daltons have been identified (Jacobs et al., 1979; Yip et al., 1980; Wisher et al., 1980). The identity of the two polypeptides has been confirmed by chemical crosslinking to radiolabeled insulin and by purification of the receptor by affinity chromatography.

In cases where it has proved impractical to assay for activity at each step, protein purification has been aided by tagging a fraction of the molecules with a photoaffinity reagent (e.g. the lactose carrier of *Escherichia coli:* Newman et al., 1981; and the β-adrenergic receptor of frog erythrocytes: Shorr et al., 1982).

1.2.2. The identification of a component of a multisubunit system

The binding of ligands to the β-adrenergic receptor of plasma membranes stimulates adenylate cyclase activity in a process that requires GTP. The existence of a separate GTP-binding protein (42,000 daltons), besides the hormone binding component and the cyclase, was confirmed by photoaffinity labeling with γ-(4-azidoanilino)-GTP (Pfeuffer, 1977). Recent purification of the GTP binding protein has confirmed the existence of a 42,000 dalton subunit that is the substrate for ADP-ribosylation by cholera toxin and NAD.

In another case, two ribosomal proteins at the peptidyl transferase site were derivatized with photolabile aminoacyl-tRNA analogs (Hsiung et al., 1974; Hsiung and Cantor, 1974).

1.2.3. The identification of a ligand binding site within a polypeptide

In favorable cases a labeled receptor may be degraded by proteolysis or chemical cleavage to identify the region of the polypeptide chain labeled by a photoaffinity reagent (Chapter 3). Occasionally the site of labeling has been precisely mapped. Kerlavage and Taylor (1980) were able to locate a single labeled tyrosine when the regulatory subunit of cAMP-dependent protein kinase II was labeled with 8-azido-cAMP.

1.2.4. The use of photoaffinity labeling in studies of function

Besides providing structural information, photoaffinity labeling is useful in studies of the function or cellular metabolism of proteins, although the full potential of this aspect of the method has not yet been realized. Covalent attachment of a ligand to a receptor can block the binding site to fresh ligand or, if the site is allosteric, fix a protein in an active or inactive conformation. Staros and Knowles (1978) have selectively inactivated the dipeptide transport system of *E. coli* by irradiating living cells with a photoaffinity reagent. Galardy et al. (1980) have produced irreversible activation of pancreatic secretion with a photolabile analog of cholecystokinin. More recently, persistant activation of steroidogenesis occurred

when adrenocortical cells were photolysed with an arylazido-ACTH derivative (Ramachandran et al., 1981), and irreversible activation of adenylate cyclase has been produced with photoactivatable derivatives of parathyroid hormone and glucagon (Draper et al., 1982; Demoliou-Mason and Epand et al., 1982). Schaltmann and Pongs (1982) were able to follow the movement of a photoaffinity labeled steroid hormone receptor from the cytoplasm to the nucleus of the cell, and Berhanu et al. (1982) have observed the fate (internalization followed by proteolytic cleavage) of a covalent insulin–receptor complex that had been formed photochemically.

1.2.5. Time-dependent photoaffinity labeling

Park et al. (1982a,b) have examined the mechanism of promoter selection by *E. coli* RNA polymerase on T7 bacteriophage DNA, using rapid mixing and flash photolysis (which activates unmodified DNA). Different delay intervals before photolysis were used with a resolution of $\sim$ 30 ms, limited by the mixing time. By determining the distribution of the enzyme along the DNA at different timepoints, and by showing that the enzyme exchanged relatively slowly between DNA molecules, it was demonstrated that the promoter is found by linear diffusion along the DNA, rather than by random collisions.

1.2.6. Further uses of photogenerated reagents

Extensions of the technique of photoaffinity labeling have proved useful in almost every area of biochemistry and molecular biology. In the membrane field, hydrophilic photoactivatable reagents have been used to label peripheral membrane proteins and the exposed surfaces of integral proteins while hydrophobic reagents have been used to derivatize the regions of integral proteins associated with the lipid bilayer (Chapter 6). For example, Khorana's group has developed a collection of phospholipids containing photoactivatable groups which, in membranes, are buried within the bilayer (Radhakrishnan et al., 1980; Robson et al., 1982). On activation the photolabile lipids react with neighboring lipids and proteins. Using such

reagents the organization of protein complexes with respect to the lipid bilayer can be investigated.

Photochemical reagents have been devised for crosslinking both soluble and particulate proteins (Chapter 5). In a recent study, Johnson et al. (1981) were able to crosslink radiolabeled glucagon to its receptor, merely by adding a bifunctional reagent that first reacted with amino groups and could subsequently be induced to form crosslinks by photolysis.

Underivatized nucleic acids will partake in intermolecular photochemical reactions and there is an extensive literature on the use of photochemical crosslinking in the nucleic acid field. Recent work has been directed towards the development of selective methods so that the investigator can have more control over the site and nature of the crosslinks. Cantor and his coworkers are developing psoralen derivatives for protein–nucleic acid crosslinking (Cantor, 1980). Psoralens intercalate into double-stranded nucleic acids and on irradiation they form crosslinks, by addition to pyrimidine bases, in sequences where purines and pyrimidines alternate. Irradiation at the long wavelength edge of their absorption results in monoadducts rather than crosslinks. The appropriate psoralen derivative may first be attached chemically to a protein of interest and the derivative can be used for labeling nucleic acid molecules that associate with the protein.