The Mechanism of Photoaffinity Labeling
(active sites of proteins/photolysis/acyethylcholinesterase)

ARNOLD E. RUOHOL, HANSRUEDI KIEFER*, PHOEBE E. ROEDER, AND S. J. SINGER†

Department of Biology, University of California at San Diego, La Jolla, Calif. 92037

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ABSTRACT Photoaffinity labeling is a recently introduced method for covalently binding chemical tags to the active sites of protein molecules, which is potentially capable of very great specificities of labeling. A labeling reagent is used that is converted by photolysis to an extremely reactive intermediate. According to the expected mechanism, the reagent molecules that are specifically and reversibly bound to the active site at the instant of photolysis react irreversibly in the site before they can dissociate from the site. In two such reagent-protein systems studied in this paper, however, it is shown that, while the usual criteria photoaffinity labeling appears to have occurred, the expected mechanism in fact does not hold. This was discovered in experiments with scavengers present in the mixtures that were photolyzed. The general properties of, and criteria for, photoaffinity labeling reactions are discussed in the light of these findings.

In the usual method for affinity labeling of the active site of a protein (1–3), a small-molecule labeling reagent R-X is used, where R is capable of being specifically and reversibly bound to the active site, and X is a chemically reactive group. An important variant of this method is photoaffinity labeling (4, 5) in which a labeling reagent R-P is used, where P now is a group that is chemically unreactive in the dark, but upon photolysis is converted to an extremely reactive intermediate P*. In principle, P* may react to form a covalent bond with some group in the active site before R-P* can dissociate from the site. Since the introduction of photolabile reagents into site-labeling studies by Westheimer and his colleagues (6), several investigations (4, 5, 7–9) have used this approach. In initial studies from this laboratory (7), we showed that the acetylcholinesterase (AChE; EC 3.1.1.7) activity of intact human erythrocyte membranes and the acetylcholine receptor (AChR) activity of frog sartorius muscle could be specifically and irreversibly inactivated by photolysis in the presence of either of two aryl azides, 4-azido-2-nitrobenzyltrimethylammonium (HK-83) or 4-azido,2-nitrobenzyltriethylammonium (HK-68) (Fig. 1). Aryl azides are converted upon photolysis to highly reactive aryl nitrenes (4). We therefore prepared [H]labeled HK-83 for quantitative labeling studies aimed at the eventual identification and isolation of AChR proteins. In the course of further experiments, however, we discovered that the chemistry of photoaffinity labeling can be more complicated than has been realized, with critical consequences for the labeling process. These experiments, the complications that were observed, and the general criteria developed to recognize these complications are reported in this paper. A preliminary account of these studies has been published (10).

MATERIALS AND METHODS
[3H]Diisopropylfluorophosphate was purchased from Amershams-Searle (Arlington Heights, Ill.) at a specific activity of 3.3 Ci/mmol and was diluted to 1 Ci/mmol with nonradioactive diisopropylfluorophosphate (Sigma Chemical Co.) for use in labeling experiments. [3H]HK-83, HK-37, and HK-68 (Fig. 1) were prepared by methods to be described in detail elsewhere. [3H]HK-83 used in these experiments had a specific activity of 190 Ci/mmol. Dnp-e-aminocaproate was prepared by the reaction of 2,4-dinitrofluorobenzene and e-aminocaproic acid, essentially by a published procedure (11). Dnp-acetic acid was purchased from K and K Laboratories, Plainview, N.Y., and p-aminobenzoate from Matheson, Coleman and Bell, Los Angeles, Calif. Pure antibodies against Dnp prepared in rabbits were prepared essentially by the method of Farah et al. (12), as in our earlier studies (13). Normal rabbit IgG and crystalline bovine-serum albumin were obtained from Miles-Pentex, Kanakee, Ill., and RNase (5X crystallized) from Calbiochem, San Diego, Calif. Human erythrocyte membranes were isolated by the method of Dodge et al. (14), and stored in isotonic sodium phosphate buffer (pH 7.4).

Photoaffinity labeling experiments were done in an apparatus that had a HBO 200 W super-pressure mercury lamp (Osram) as a light source mounted in a Bausch and Lomb lamp housing. This was connected to a Bausch and Lomb high-intensity monochromator, 33-86-01 for the ultraviolet region, or 33-86-02 for the visible, fitted with quartz no. 1 lenses, variable slits, and a shutter to control the time of photolysis. The sample to be irradiated was placed in a quartz cuvette of 1-cm path length, or a double-chambered cell each of 5-mm path length, centered in the light beam.

For experiments with intact erythrocyte membranes, 1.0 ml of a suspension containing 1 mg of membrane protein and the desired concentrations of azide and other reagents in the isotonic phosphate buffer was photolyzed with stirring at 365 nm at room temperature for 90 sec. After photolysis, the samples were immediately diluted to 30 ml with the isotonic phosphate buffer at 0°C, and washed five times by centrifugation at 15,000 X g for 5 min. The pellets of membrane were then usually suspended in 1.0 ml of the isotonic phosphate buffer; 10 μl were removed for assay of AChE activity.

Abbreviations: AChE, acetylcholinesterase; AChR, acetylcholine receptor; Dnp, 2,4-dinitrophenyl; IgG, normal immunoglobulin G.

* Present address: The Basel Institute for Immunology, Basel, Switzerland.
† To whom reprint requests should be addressed.
TABLE 1. Inactivation and labeling of erythrocyte acetylcholinesterase

<table>
<thead>
<tr>
<th>Reagent (M)</th>
<th>% Esterase activity retained</th>
<th>Labeling</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cpmp/ml per A₂₅₀</td>
</tr>
<tr>
<td>[³H]DFP (10⁻⁴)</td>
<td>13</td>
<td>5730</td>
</tr>
<tr>
<td>[³H]DFP (10⁻⁴), HK-37, 10⁻⁴</td>
<td>110</td>
<td>2670</td>
</tr>
<tr>
<td>[³H]DFP (10⁻⁴), HK-88 (10⁻⁴) (dark)</td>
<td>95</td>
<td>3330</td>
</tr>
<tr>
<td>[³H]HK-83* (10⁻⁴)</td>
<td>18</td>
<td>4.7 × 10⁴</td>
</tr>
<tr>
<td>[³H]HK-83* (10⁻⁴), HK-37 (10⁻⁴)</td>
<td>95</td>
<td>4.9 × 10⁴</td>
</tr>
</tbody>
</table>

DFP, diisopropylfluorophosphate.

* Mixture photolyzed.

(15); 20 μl were mixed with 10 ml of a scintillation solution containing 9% NCS (Amersham/Searle) and 4% Liquifluor (New England Nuclear Corp.) in toluene for radioactive counting; and 0.8 ml was added to 1.2 ml of 1% aqueous sodium dodecyl sulfate for determination of the absorbance at 280 nm, a measure of the protein concentration. For the experiments with the antibodies against Dnp, 0.8 ml of a solution containing 6.25 μM antibodies, 25 μM [³H]HK-83, and the desired concentrations of scavenger or protector in 80 mM phosphate buffer (pH 7.4) was placed in the rear chamber of the double-chambered quartz cuvette. To the front chamber was then added buffer or solutions of scavenger or protector, as absorption controls. Photolysis was done at 365 or 324 nm for 90 sec. Photolyzed samples were then dialyzed overnight against 10 mM Dnp-p-aminocaproate to displace other noncovalently bound reagents from the antibody active sites, and then exhaustively against the phosphate buffer. Tritium covalently bound to the antibody was measured on an aliquot of this dialyzed solution. A Beckmann LS-200B scintillation counter was used in these experiments. Measurements of light flux were made with potassium ferrioxalate (16).

RESULTS

Acetylcholinesterase

Labeling with [³H]Diisopropylfluorophosphate. DFP is an irreversible inhibitor of AChE of erythrocytes (17). Since it reacts with great specificity with activated seryl residues in the active sites of esterases, we used [³H]diisopropylfluorophosphate to provide a measure of the numbers of AChE sites available, for comparison with the results obtained with [³H]HK-83. In Table 1, it is shown that accompanying the irreversible binding of only about 7 × 10⁻⁴ μmol of [³H]-diisopropylfluorophosphate per 1.0 A₂₅₀ unit of membrane protein, there was a nearly complete inactivation of the AChE. This inactivation was prevented by the presence of the specific protector HK-37 (Fig. 1) at 10⁻⁴ M, and also by HK-83 at 10⁻⁴ M in the dark.

Labeling with [³H]HK-83. As in our previous studies with nonradioactive HK-83 (7), photolysis of AChE in the presence of 10⁻⁴ M [³H]HK-83 caused a large (82%) irreversible inactivation of the enzyme (Table 1). In the dark, however, [³H]HK-83 produced no irreversible inactivation, nor did photolysis of the erythrocyte ghost preparation itself. The irreversible inactivation was prevented by the presence of 10⁻⁴ M HK-37. However, when the radioactivity of the membrane preparations reacted with [³H]HK-83 was measured, there was about 1000 times as much label as was found with diisopropylfluorophosphate, whether or not the specific protector HK-37 was present. This finding shows that while HK-83 appeared to be capable of photoaffinity labeling of AChE, it was also reacting nonspecifically and extensively with other membrane components.

Scavenging with p-Aminobenzoate. If photoaffinity labeling occurred by the expected mechanism in a specific active site, but was accompanied by nonspecific reactions outside the site, we had earlier suggested (7) that suitable "scavengers" might be found to react with the R-P⁺ formed in free solution, and thereby eliminate, or at least greatly reduce, the extent of the

TABLE 2. Scavenging of photoaffinity labeling of erythrocyte acetylcholinesterase

<table>
<thead>
<tr>
<th>Exp. no.</th>
<th>HK-37 (Protector) (M)</th>
<th>Scavenger, conc.</th>
<th>% Esterase activity retained</th>
<th>Labeling μmol/ml per A₂₅₀</th>
<th>×10⁴</th>
<th>A₁₆₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>24</td>
<td>5.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>0</td>
<td>PAB (10⁻⁴ M)</td>
<td>95</td>
<td>0.54</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>0</td>
<td>PAB (10⁻⁴ M)</td>
<td>100</td>
<td>0.61</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2*</td>
<td>0</td>
<td>0</td>
<td>40</td>
<td>2.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>0</td>
<td>BSA (25 mg/ml)</td>
<td>99</td>
<td>0.49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>0</td>
<td>BSA (25 mg/ml)</td>
<td>97</td>
<td>0.48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>0</td>
<td>RNase (25 mg/ml)</td>
<td>90</td>
<td>0.78</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>0</td>
<td>RNase (25 mg/ml)</td>
<td>91</td>
<td>0.76</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

† In all components of this experiment, a cell containing 25 mg/ml of protein [either bovine-serum albumin (BSA) or RNase] was placed in front of the reaction cell during photolysis.
nonspecific reactions. After initial trials with soluble proteins added to the erythrocyte ghost suspensions, we found that p-aminobenzoate was an effective scavenger for the reactive photolysis product of HK-83. Thus, it was indeed found (Table 2) that the radioactivity irreversibly bound to the ghosts after photolysis of $10^{-3}$ M $[^{3}H]$HK-83 was reduced about 10-fold in the presence of $10^{-3}$ M p-aminobenzoate. However, an unexpected result was obtained: the presence of the p-aminobenzoate prevented the photolytic inactivation of the AChE by HK-83 (Table 2, column 4).

We had earlier shown (7) that HK-68 also acts as a photoaffinity labeling reagent for the AChE activity of erythrocytes. The presence of $10^{-3}$ M p-aminobenzoate also prevented the irreversible photoinactivation by this aryl azide (data not shown).

In double-cell experiments, with the p-aminobenzoate in the front cell and the mixture of $[^{3}H]$HK-83 and erythrocyte ghosts in the rear cell, the amount of radioactivity irreversibly bound to the ghosts was essentially the same as with buffer in the front cell (Table 3). Thus, the effect of $10^{-3}$ M p-aminobenzoate, when present in the mixture, can not simply be ascribed to the absorbance of the photolytic radiation.

p-Aminobenzoate, prephotolyzed HK-83, and a prephotolyzed mixture of p-aminobenzoate and HK-83, were each examined for reversible inhibition of AChE activity (Fig. 2). While the solution of prephotolyzed HK-83 itself contained an effective inhibitor, neither p-aminobenzoate nor the photolyzed mixture of p-aminobenzoate and HK-83, were inhibitory.

**Scavenging with Proteins.** Soluble proteins were effective scavengers in this system. In the presence of 25 mg/ml of bovine-serum albumin or RNase, the total amount of radioactive label covalently attached to the membranes by photolysis of $[^{3}H]$HK-83 was reduced about 5- or 4-fold, respectively (Table 2). This reduction could not be ascribed simply to absorption of the photolyzing radiation by the proteins, since actinometer measurements showed that even at 50 mg/ml, the proteins absorbed only 20% of the incident light at 365 nm. Furthermore, the presence of the protein scavengers also prevented the irreversible inactivation of AChE by photolysis of HK-83 (Table 2).

**Antibodies against Dnp.** Because these scavenging results were unexpected, we wanted to find out if they were characteristic of AChE only, or whether the active sites of other proteins behaved similarly towards HK-83. The 2-nitro,4-azidophenyl portion of HK-83 has a weak but specific affinity for antibodies against Dnp, and the photoaffinity labeling of these pure rabbit antibodies, and of normal rabbit IgG as a control, was therefore undertaken.

The results are shown in Table 4. $[^{3}H]$HK-83 at 25 μM did covalently label antibodies against Dnp upon photolysis but not in the dark. Part of this covalent label was specific for the active sites of the antibodies, as indicated by the fact that the specific protector, Dnp-$\alpha$-aminocaproate, at the low concentrations of 20–25 μM, reduced the amount of covalently bound label to that taken up by normal IgG under the same conditions (Exps. 2 and 3, Table 4). In the presence of $10^{-3}$ M p-aminobenzoate, however, the amounts of label on both the antibodies against Dnp and the normal IgG were markedly reduced, suggesting that the specific inactivation of the antibodies was largely prevented by p-aminobenzoate. This suggestion was confirmed by reversible binding measurements of the treated antibodies.

![Fig. 1. Structures of reagents used in this study.](image)

![Fig. 2. Reversible inhibition of the enzymic activity of erythrocyte acetylcholinesterase: by prephotolyzed HK-83 (○); by p-aminobenzoate (○); and by a prephotolyzed mixture of HK-83 and p-aminobenzoate (●). In the first and last cases, the concentration quoted is that of the original azide in solution before photolysis.](image)

![Fig. 3. Schematic representation of the mechanisms of ordinary affinity labeling and photoaffinity labeling.](image)
TABLE 4.  Photoaffinity labeling of antibodies against Dnp and normal IgG with [3H]HK-83

<table>
<thead>
<tr>
<th>Exp. no.</th>
<th>Reagents* added, mM</th>
<th>mol of label/mol of anti-Dnp</th>
<th>mol of label/mol of normal IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>—</td>
<td>0.70</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>PAB, 0.025</td>
<td>0.58</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>PAB, 1.0</td>
<td>0.35</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>PAB, 10</td>
<td>0.16</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>Prephotolyzed†</td>
<td>0.01</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Dark‡</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>2‡</td>
<td>Dnp-ε-aminocaproate, 0.020</td>
<td>0.57</td>
<td>0.65</td>
</tr>
<tr>
<td>3‡</td>
<td>Dnp-ε-aminocaproate, 0.025</td>
<td>0.63</td>
<td>0.36</td>
</tr>
<tr>
<td>4</td>
<td>Dnp-acetic acid, 1.0</td>
<td>0.74</td>
<td>0.37</td>
</tr>
</tbody>
</table>

PAB, p-aminobenzoate.

* In each case, except where indicated, 6.25 μM protein and 25 μM [3H]HK-83 were present, along with indicated reagents, during photolysis.
† In this case, [3H]HK-83 was first photolyzed for the 90-sec period, then added to the protein solution.
‡ Mixture of protein and [3H]HK-83 was kept dark.
§ Larger concentrations of the protector Dnp-ε-aminocaproate led to appreciable absorption of the photolyzing radiation.

An interesting observation is that Dnp-acetic acid, which was expected to be a specific protector of the active sites of antibody against Dnp, acted instead primarily as a scavenger at 1 mM. This is shown by the fact that it markedly reduced the amount of label irreversibly bound to normal IgG (Exp. 4, Table 4), as well as to the antibodies. On the other hand, Dnp-ε-aminocaproate at 20–25 μM did not reduce the uptake of label by normal IgG (Exps. 2 and 3), and hence the decreased binding that it produced with the antibodies is a true protection effect.

DISCUSSION

The mechanisms of ordinary affinity labeling, with a reagent R-X containing a chemically X of ordinary reactivity, and of photoaffinity labeling with a photolabile reagent R-P, are schematically represented in Fig. 3. In order for true photoaffinity labeling to occur, the rate constant \( k_{p*2} \) must be significantly larger than \( k_{p^*} \). Under such circumstances, the photolytic intermediate R-P*, which is produced while the molecule R-P is reversibly bound to the active site, will generally react covalently within the site before it can dissociate from the site. Those molecules of R-P* that are produced by photolysis of R-P in free solution may then react with the solvent or with added scavengers, and only minimally with the protein. The specificity of the labeling of the active site in question might thus be very large (7). On the other hand, if \( k_{p^*} > k_{p*2} \), then what can be called pseudo photoaffinity labeling may result. R-P* present in free solution may bind reversibly to, and dissociate from, the active site many times before it (or its product) reacts to form a covalent bond within the active site. In principle, therefore, pseudo photoaffinity labeling is basically the same as ordinary affinity labeling, with the now trivial difference that the labeling reagent R-P* is produced in a photolytic reaction. In particular, the specificity of the labeling reaction would very likely be similar for pseudo photoaffinity labeling and ordinary affinity labeling, other things being equal. This is because the extent of nonspecific labeling of the protein might be equally extensive in both cases, if R-P* does not react rapidly with the solvent. Addition of a scavenger would not be helpful in this case, because by reacting with and using up the R-P* in free solution, it would equally diminish the extent of both the site-specific and nonspecific reactions of R-P*.

We had earlier shown (7) that the reaction of HK-83 and AChE bound to erythrocyte ghosts appeared to satisfy the criteria ordinarily used for true photoaffinity labeling. No irreversible reaction occurred without photolysis; the irreversible inactivation produced by photolysis of HK-83 in the presence of AChE was prevented by specific protectors of the AChE active site; and prephotolysis of HK-83 itself, followed within minutes by its addition to AChE, produced no irreversible inactivation. However, the experiments reported in this paper strongly indicate that the reaction is actually a pseudo photoaffinity labeling process. No other satisfactory explanation occurs for the fact that both small-molecule and large-molecule scavengers prevent the irreversible photolytic inactivation of AChE by HK-83. Several trivial explanations have been eliminated: (a) p-aminobenzoate and the protein scavengers do not simply absorb the photolyzing radiation. (b) The scavengers do not bind reversibly to the AChE active site, nor do the products of their photolytic reaction with HK-83; therefore, a displacement of still-not-photolyzed HK-83 from the AChE sites during the photolysis experiment cannot be the explanation of the scavenging results.

Furthermore, the labeling experiments with HK-83 and pure antibodies against Dnp are consistent with the findings with AChE. The photolabeling reaction with [3H]HK-83 is at least partly specific for the antibody sites, since the labeling is reduced in the presence of a low concentration of a specific protector, Dnp-ε-aminocaproate. The extent of labeling by [3H]HK-83 of either the antibodies or normal IgG is markedly reduced in the presence of the scavenger, p-aminobenzoate. Furthermore, the equilibrium association constant for the reversible binding of HK-83 to antibodies against Dnp (in the dark) is about \( 4 \times 10^3 \text{ M}^{-1} \) (P. E. Roeder and S. J. Singer, unpublished experiments), which means that at the HK-83 and antibody concentrations used in the photolabeling experiments, less than 8% of the antibody sites were occupied by HK-83. In order for specific reaction to have occurred in the active site, a photolytic intermediate formed in free solution (rather than one formed in the site) must therefore have been the labeling reagent. A difference between the AChE and anti-Dnp sites is that while in the former case the bound nitrene N atom of photolyzed HK-83 might protrude from the active site into the aqueous medium, in the latter case that N atom must be facing into the active site. Nevertheless, in both cases the results of the scavenging experiments with PAB were the same.

Our conclusion is that despite the fact that the photolabeling reactions of HK-83 with AChE, AChR (7), and antibodies against Dnp satisfy the usual criteria for true photoaffinity labeling, the scavenging results indicate that the reaction occurs by ordinary affinity labeling. It is therefore
legitimate to ask whether in other photoaffinity labeling experiments was the mechanism of the reaction observed indeed true photoaffinity labeling? Until the appropriate scavenging experiments are performed, however, the answer is uncertain. We have performed photolabeling experiments (P. E. Roeder and S. J. Singer, to be published) with antibodies against Dnp and with the aryl azide (4) 4-azido-2-nitrophenyl-e-N-l-lysine (NAP-lysine, Fig. 1), which suggest that in this case at least, true photoaffinity labeling appears to occur. Under given reaction conditions with [3H]NAP-lysine, antibodies against Dnp were labeled to about 15-times the extent of normal IgG (in contrast to HK-83, Table 3) and the scavenger p-aminobenzoate at 10^{-4} M did not markedly reduce the extent of labeling of the antibodies nor of the normal IgG.

At this time, it is not feasible for chemists to predict whether a given photolyzable reagent will satisfy the condition $k_{p_{*2}} > k_{p_{*1}}$ with a particular protein to be photoaffinity labeled. The relatively long life-times of simple aryl nitrenes (18), which correspond to small values of $k_{p_{*2}}$, might therefore be a liability in photoaffinity labeling experiments, rather than an asset as suggested by Fleet et al. (19). The smaller the value of $k_{p_{*2}}$, the more likely true photoaffinity labeling will occur, other things being equal. This generally means that the larger the reversible binding affinity of the photolyzable reagent for the active site, the better, other things being equal.

In view of the present inability to predict a priori the properties of a given reagent–protein system in photoaffinity labeling experiments, it is of considerable importance to determine early in an investigation whether or not true affinity labeling occurs. This information bears on the degree of specificity of labeling that can be expected, and would be useful to have before time and money is invested in making a highly radioactive form of the reagent. Furthermore, one can be misled in the interpretation of protection experiments, if pseudo rather than true photoaffinity labeling occurs, because the putative protector may actually function as a scavenger. This was demonstrated in our experiments when Dnp-acetic acid was used in the photolabeling experiments of antibodies against Dnp by $\text{[PH]HK-83}$ (Exp. 4, Table 3). The data of Exp. 4 with the antibodies against Dnp alone might be interpreted to indicate a protection of the antibodies by Dnp-acetic acid, but the fact that Dnp-acetic acid also reduced the amount of label attached to normal IgG indicates that, instead, it is acting as a scavenger of the photoreactive species produced from HK-83.

For these and other reasons, we therefore recommend that scavenging experiments, with suitable reagents such as PAB, be incorporated into the design of photoaffinity labeling experiments. With proper precautions to show that the scavenger does not simply absorb the photolyzing radiation, or act as (or produce) a reversible inhibitor of the active site in question, the prevention or reduction by the scavenger of the inactivation produced in the photolabeling reaction is prima facie evidence that the mechanism of the labeling reaction is not true photoaffinity labeling. On the other hand, where scavengers have no such effect, and true photoaffinity labeling apparently occurs, the scavengers should subsequently be useful in minimizing the extent of nonspecific labeling of the proteins and other components during the photoaffinity labeling reaction.

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