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p-Azidophenacyl Bromide, a Versatile Photolabile Bifunctional Reagent. Reaction with Glyceraldehyde-3-phosphate Dehydrogenase†

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ABSTRACT: The synthesis of the photochemically labile bifunctional reagent *p*-azidophenacyl bromide (**1**) is described. This compound may be covalently attached to a known site of an enzyme or other macromolecule by nucleophilic displacement at the α-bromo ketone moiety. Subsequent irradiation of the bound reagent gives a nitrene which may insert into a second portion of the enzyme forming a cross-link. Reagent **1** proved to be an excellent inhibitor of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase.

The use of specificially designed aryl azides as photochemical labeling reagents for investigating binding sites of macromolecules is an attractive concept (Knowles, 1972). However, recent results in various laboratories including our own have shown that there are severe problems and limitations with the method. When we used the labeling reagent 3-azidopyridine adenine dinucleotide, a loosely bound (K_i *ca.* 10^{-4} *M*) inhibitor of yeast alcohol dehydrogenase (YADH),[1] to probe the active site of this enzyme (Hixson and Hixson, 1973), much nonactive site labeling appeared to occur. Much of this random labeling is likely a result of the loose binding of the inhibitor to YADH. However, poor binding is not the only factor, for we have recently obtained strong evidence that when the reagent *p*-azidobenzenesulfonamide, a tightly bound inhibitor (K_i *ca.* 10^{-6} *M*) of bovine erythrocyte carbonic anhydrase (CA), is irradiated under conditions where >99% of the reagent is initially located (noncovalently) at the active site of CA, covalent labeling of the protein occurs largely at points removed from the active site (A. Lowrie and S. Burroughs, unpublished results).

This random labeling problem is almost certainly in large part a result of the rather low reactivity of aryl nitrenes. They do not react "instantaneously" upon generation, but instead have a sufficiently long lifetime that they may diffuse out of the binding site and react with more remote portions of the protein (Knowles, 1972). Other workers, notably Ruoho et al. (1973) and Richards et al. (1974), have encountered and discussed this problem in their work with other systems. Our conclusion has been that the use of noncovalently bound aryl azides as photochemical probes of binding sites of macromolecules is risky and apt to provide ambiguous information. Consequently we are turning to the use of bifunctional photochemical reagents which may be bound covalently at a known locus in the macromolecule prior to photolysis.

In this direction we have prepared the compound *p*-azidophenacyl bromide (**1**). This molecule contains the photochemically labile azide group along with a reactive α-bromo ketone moiety. α-Halo carbonyl compounds are known to label nucleophilic amino acids such as cysteine in proteins. Therefore reagent **1** should be particularly useful for investigating the active sites of the large number of sulfhydryl enzymes—those which possess a particularly reactive cysteine residue at the active site that may be selectively derivatized—and the compound was designed for this purpose. When **1** is irradiated after being covalently attached to the active site of the enzyme, any portion of the protein chain labeled by the resulting nitrene must be close in three-di-

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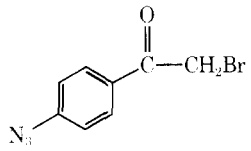
[1] Abbreviations used are: CA, bovine erythrocyte carbonic anhydrase; GPDH, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase; YADH, yeast alcohol dehydrogenase.

mensional space to the residue to which **1** was originally attached. The labeling information is therefore unambiguous.



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Since our initial report on the use of reagent **1** as a labeling reagent for enzyme active sites (Hixson et al., 1973), Schwartz and Ofengand (1974) have published work in which they successfully used **1** to study the nature of the ribosomal binding site of *E. coli* $tRNA^{Val}$. Since **1** should be highly useful in investigating a large number of other biological systems as well, we wish to make available our synthesis of this reagent in both unlabeled and ^{14}C-labeled form. We also present our studies to date on the interaction of **1** with rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (GPDH), a typical sulfhydryl enzyme.

Experimental Section

Methods. Melting points were taken on a Mel-Temp apparatus and are uncorrected. Ultraviolet spectra were taken on a Cary 14 spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian A-60 or a Perkin Elmer R12A instrument. Radioactivity was measured with a Packard Model 3310 TriCarb scintillation spectrometer using a cocktail made from 168 ml of Liquifluor (New England Nuclear), 1832 ml of toluene, and 2000 ml of absolute ethanol. Microanalyses were carried out by the University of Massachusetts Microanalytical Laboratory.

Large Scale Synthesis of p-Azidophenacyl Bromide (**1**). A 6.00-g (37.3 mmol) portion of *p*-azidoacetophenone (Pochinok and Kalashnikova, 1951; Hepher and Wagner, 1960) was dissolved in 20 ml of anhydrous ether plus 10 ml of dioxane (sodium-dried) in a three-necked round-bottomed flask containing a stir bar and equipped with a stopper, a serum cap, and a condenser with a Drierite drying tube. The flask was kept in a cold water bath at *ca.* 15°. Then 1.91 ml (37.3 mmol) of bromine was added dropwise via syringe. The bromine decolorized immediately. After 20 min the reaction mixture was diluted with water and ether, and the ether layer was separated. The water layer was extracted again with ether, and the combined ether layers were washed twice with water and once with saturated sodium chloride solution, dried ($MgSO_4$), filtered, and evaporated. Crystallization from ether gave a first crop of 5.25 g (58%) of *p*-azidophenacyl bromide, mp 66–69°. Recrystallization gave a mp 68–70°. The spectral data were: ir (KBr) 2166 (N_3) and 1666 (CO) cm^{-1}; NMR ($CDCl_3$) δ 4.35 (s, 2, CH_2), 7.04 (d, 2, arom), and 7.96 ppm (d, 2, arom); uv (2% methanol in H_2O) λ_{max} 300 nm (ϵ 2.01 × 10^4); uv (CH_3OH) λ_{max} 292.5 nm (ϵ 2.08 × 10^4). On thin-layer chromatography on silica gel plates **1** had an R_f 0.39 (R_f of *p*-azidoacetophenone 0.31) when 1:3 diethyl ether–Skellysolve B was the eluent. Anal. Calcd for $C_8H_6BrON_3$: C, 40.02; H, 2.52; N, 17.50. Found C, 40.33; H, 2.74; N, 17.66.

If excess bromine was used in the bromination, a large amount of an incorrect product, presumably dibrominated *p*-azidoacetophenone, was formed.

Synthesis of [carbonyl-^{14}C]-p-Azidophenacyl Bromide. A suspension of 0.0163 g (0.119 mmol) of [*carboxyl*-^{14}C]-*p*-aminobenzoic acid (ICN Pharmaceuticals, Inc., lot no. 561062, 8.4 Ci/mol) and 0.0137 g (0.100 mmol) of unlabeled *p*-aminobenzoic acid in 5 ml of 11% sulfuric acid was cooled in an ice bath. A solution of 0.0477 g (0.692 mmol) of sodium nitrite in 0.3 ml of water, cooled to 0°, was added dropwise over 1 min to the stirred suspension. After stirring the mixture for 0.5 hr in the ice bath, a 5-ml portion of ether was added. A solution of 0.0730 g (1.12 mmol) of sodium azide in 0.3 ml of water was added dropwise quickly to the reaction mixture. The reaction was stirred 15 min at 0°, then 0.5 hr at room temperature. The ether layer was decanted. The remaining water layer was stirred with 5 ml of ether and the ether was decanted; then 3 ml of ether, again decanted. The combined ether layers were stirred with 3–4 ml of saturated sodium chloride solution, decanted, dried ($MgSO_4$), and filtered into a 5-ml round-bottomed flask. Removal of the ether with a stream of nitrogen afforded 0.032 g (0.197 mmol, 90%) of *p*-azidobenzoic acid.

A 1.0-ml portion of thionyl chloride was added to the [^{14}C]-*p*-azidobenzoic acid in the 5-ml round-bottomed flask containing a stir bar. The solution was refluxed 0.5 hr and then cooled to room temperature. A micro distillation head was substituted for the condenser. The thionyl chloride was distilled off under low vacuum (ca. 20 mm). A 0.5-ml portion of dry benzene was added and distilled; this process was repeated twice to ensure thionyl chloride removal. The solid acid chloride was kept under vacuum until used (~2 hr).

Dimethylcadmium was then prepared. A solution of 3 ml of ether and 0.75 ml (2.25 mmol) of methylmagnesium bromide solution (Ventron, 3 *M* in ether) in a three-necked round-bottomed flask equipped with a condenser and a mechanical stirrer was kept under nitrogen and cooled in an ice bath. Then 0.2066 g (1.13 mmol) of cadmium chloride (dried at 110° and stored in a desiccator over Drierite) was added to the methylmagnesium bromide solution in one portion. The reaction flask was transferred to an oil bath and the mixture was refluxed and stirred for 20 min and then cooled to room temperature. A Gilman test was negative. A distilling head was added to the flask and most of the ether was distilled over while stirring continued. A 7-ml portion of dry benzene was added to the reaction mixture and distillation was continued until the temperature of the distilling vapors was about 70°. The distillation head was removed and the reaction mixture containing dimethylcadmium (under N_2) was cooled in an ice bath.

The acid chloride prepared above was dissolved in 0.3 ml of dry benzene and transferred quickly via syringe to the stirred dimethylcadmium solution. The acid chloride flask was rinsed twice with 0.3-ml portions of benzene and these rinses were added to the dimethylcadmium flask as well. The reaction mixture was stirred at room temperature for 0.5 hr. Then several pieces of ice were added to the reaction mixture followed by the sequential addition of 2 ml of 2 *N* HCl, 3 ml of ether, and then additional water. The ether–benzene layer was decanted. The water layer was stirred twice with 3-ml portions of ether which were decanted. The combined organic layers were stirred with ca. 5 ml of saturated sodium bicarbonate solution, then dried ($MgSO_4$), filtered, and blown dry under nitrogen.

The crude product was chromatographed on a 1 × 30.2 cm column of deactivated (10% H_2O) silica gel with 1:10 ether–Skellysolve B as eluent to give 0.0185 g of [^{14}C]-*p*-azidoacetophenone.

The 0.0185 g (0.115 mmol) of [*carbonyl*-^{14}C]-*p*-azidoacetophenone was converted to *p*-azidophenacyl bromide following the procedure used for the large scale preparation using 0.5 ml of ether plus 0.25 ml of dioxane as solvent and 5.9 μl (18.4 mg, 0.115 mmol) of bromine.

The crude product was chromatographed on a 1 × 30.4 cm column of deactivated (10% H_2O) silica gel with 1:5 ether–Skellysolve B as eluent to give 0.0185 g (0.077 mmol) of [*carbonyl*-^{14}C]-*p*-azidophenacyl bromide (35.2% overall yield based on *p*-aminobenzoic acid) with a specific activity of 4.57 Ci/mol. The product was stored in a vial over Drierite at −20°.

Stability of p-Azidophenacyl Bromide. Since [^{14}C]-*p*-azidophenacyl bromide was to be added as a methanol solution to the enzyme, the stability of the inhibitor in methanol was of interest. One solution ca. 5×10^{-3} *M* in *p*-azidophenacyl bromide in methanol was kept at room temperature in the dark for 2.5 weeks and a second such solution was stored at −20° in the dark for 2.5 weeks. The OD_{280}/OD_{300} ratio of both solutions was then noted. The solution kept at room temperature gave a ratio of 1.04 while the solution kept in the freezer retained the original ratio of 0.88. Other solutions stored in the freezer longer than 4 months retained this ratio of 0.88.

Glyceraldehyde-3-phosphate Dehydrogenase (GPDH). Rabbit muscle GPDH (EC 1.2.1.12) was purchased from Sigma as a crystalline suspension in ammonium sulfate, lots 42C-9571-95 (14 mg of protein/ml, 105 units/mg of protein) and 91C-9530 (10 mg of protein/ml, 140 units/mg of protein). The enzyme was prepared by centrifuging an aliquot for 10 min at 20,000 *g* at 5°. The precipitate was dissolved in an appropriate amount of buffer and recentrifuged for 10 min at 20,000 *g* at 5°. The supernatant was used. An ϵ_{280} of 1.46×10^5 was used to calculate protein concentration (Fox and Dandliker, 1956).

GPDH Activity Assay Conditions. The assay solution was that of Fenselau and Weigel (1970). The assays were run on a Gilford-modified Beckman D.U. spectrophotometer at 25°.

GPDH Inhibition Studies. A solution of 2.92×10^{-5} *M* GPDH in 0.05 *M* Tris buffer (pH 7.0) at 0° was prepared. At zero time the calculated amount of a 2.88×10^{-3} *M* solution of [^{14}C]-*p*-azidophenacyl bromide in methanol was added to 0.6 ml of the enzyme solution at 0°. At given times 0.05 ml of the reaction mixture was withdrawn and added to 0.05 ml of a 0.5 *M* cysteine solution (pH 7.0). These samples were assayed for enzyme activity after being kept at 0° for at least 30 min. To determine incorporation of ^{14}C into protein a modification of the procedure of Fenselau (1970) was used. A 10-μl portion of the enzyme–inhibitor reaction mixture which had been treated with cysteine was added to a 2.4-cm Whatman glass fiber disc (GF/C) resting on a square of Parafilm. After at least 2 min 0.5 ml of 20% trichloroacetic acid was added to the disc. After at least 5 min the disc was washed in 0.2 *N* NaOH, then three times in 0.01 *N* NaOH, and finally in methanol, dried, and counted in 10 ml of scintillation fluid.

Photolysis of GPDH Labeled with [^{14}C]-p-Azidophenacyl Bromide. A 1.2-ml aliquot of a 3.68×10^{-5} *M* solution of GPDH in 0.05 *M* Tris buffer (pH 7.0) was treated at 0° in the dark with 1 equiv of a 2.88×10^{-3} *M* solution of [^{14}C]-*p*-azidophenacyl bromide in methanol. After 1.67 hr, the reaction mixture was transferred to dialysis tubing and dialyzed overnight against 0.05 *M* Tris buffer (pH 7.0), 1 m*M* in EDTA, at 5° in the dark to remove any unreacted

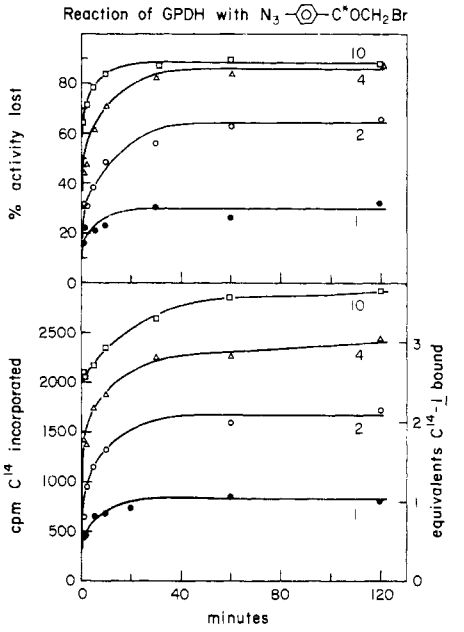

FIGURE 1: Reaction of [^{14}C]-*p*-azidophenacyl bromide with GPDH. See Experimental Section for details. (●) 1.0 equiv of inhibitor; (○) 2.0 equiv of inhibitor; (△) 4.0 equiv of inhibitor; (□) 10.0 equiv of inhibitor.

p-azidophenacyl bromide. After dialysis, the enzyme solution was transferred to a 1.0-cm diameter Pyrex tube containing a micro stir bar, and this tube was placed in a 250-ml Erlenmeyer flask containing ice–water. The sample was irradiated with light from the 350-nm bulbs of a Rayonet photochemical reactor (λ_{max} 350 nm, range 308–425 nm). Fresh ice was added to the Erlenmeyer flask as needed. Aliquots of the reaction mixture were removed at 15-min intervals for uv spectral analysis.

Control Photolysis of GPDH alone. A 3.69×10^{-5} *M* solution of GPDH in 0.05 *M* Tris buffer (pH 7.0) was dialyzed as above for the ^{14}C-labeled enzyme and was similarly photolyzed for 30 min. Aliquots (0.05 ml) of the enzyme solution before and after photolysis were added to 0.05 ml of 0.05 *M* Tris buffer (pH 7.0) and to 0.05 ml of 0.5 *M* cysteine solution (pH 7.0). After 30 min these samples were assayed for activity.

Results and Discussion

The inhibitor *p*-azidophenacyl bromide (**1**) is easily synthesized in either ^{14}C-labeled or unlabeled form. A 2.88×10^{-3} *M* methanol solution of **1** was stable when stored at −20° for more than 4 months. The ease of synthesis and thermal stability make this inhibitor a convenient, readily available reagent.

Bromide **1** proved to be an excellent inhibitor of GPDH. Upon addition of [^{14}C]**1** to the enzyme, loss of activity occurred concomitant with incorporation of ^{14}C into the protein (Figure 1). Inspection of Figure 1 reveals that 1 equiv of inhibitor led to a ca. 30% loss of activity of the GPDH and 2 equiv to a ca. 65% loss. One calculates therefore that 3–3.3 equiv of inhibitor should completely inactivate the GPDH. GPDH is a tetrameric enzyme containing one active site in each of the four identical monomers. However, inactivation of the enzyme following treatment with less than 4 equiv of sulfhydryl reagent has also been found for iodoacetate and other inhibitors (Fenselau and Weigel, 1970; Harris and Perham, 1965) known to react specifically

with the active site cysteine, so our result is not unusual. By analogy with the reaction of other α-halo carbonyl reagents with GPDH (Moore and Fenselau, 1972; Kirtley and Koshland, 1966), we assume that when 1 and 2 equiv of **1** are added to GPDH reaction occurs specifically at the active site cysteine (Cys-149) inactivating the enzyme.

Most samples of GPDH contain at least a portion of the protein in the oxidized disulfide form which is inactive and unable to react with sulfhydryl reagents. The presence of oxidized GPDH explains the failure of **1** to inactivate GPDH completely even when 10 equiv of the inhibitor were used. We did not activate the enzyme with a reducing agent prior to our studies since we wished to avoid reaction of the reducing agent with **1**. However, after the GPDH and **1** had been allowed to react for the desired time period, a large excess of cysteine was added to stop the reaction. Presumably the residual activity of the enzyme remaining after reaction with 10 equiv of inhibitor is due to disulfide reduction by the cysteine. This conclusion is supported by the fact that when the enzyme was assayed in the absence of cysteine the activity was typically 80% of that found when the assay was performed in the presence of cysteine.

The incorporation of ^{14}C into the protein paralleled loss of GPDH activity when 1 and 2 equiv of inhibitor were added. With 4 and 10 equiv the incorporation of ^{14}C paralleled loss of activity at early times but the incorporation continued to increase slowly over the entire time studied, even though the enzyme inhibition leveled off (Figure 1). It appears, therefore, that the inhibitor is initially reacting very rapidly with the active site sulfhydryl groups and that any excess inhibitor then reacts more slowly with other nucleophilic groups on the enzyme. This result indicates that in experiments where only an active site sulfhydryl group is to carry the photolabile group, an excess of the inhibitor should not be employed.

The uv spectrum of **1** itself (λ_{max} 300 nm, ϵ 2.01 $\times$ 10 ^{4}in 2% methanol in water) and of *p*-azidoacetophenone (λ_{max} 290 nm, ϵ 2.08 $\times$ 10^4 in 2% methanol in water) both show significant absorption at wavelengths well beyond 300 nm, out of the range where most proteins absorb heavily (λ_{max} ca. 280 nm). The inhibitor while bound to GPDH likewise absorbs light substantially at wavelengths greater than 300 nm. By using light of λ >300 nm one may fairly selectively irradiate the inhibitor covalently bound to the enzyme.

When a spectrum of GPDH carrying 1 equiv of the *p*-azidophenacyl moiety is compared with a spectrum of the original GPDH solution, the modified enzyme shows a larger OD 290/OD 260 ratio. During photolysis this ratio decreases as the azide is decomposed, so the changing ratio can be followed to determine the time of photolysis needed to decompose completely the azide. Under the conditions of photolysis here and with the quantities of material used, 30 min of irradiation proved sufficient. Moreover, when unmodified GPDH was photolyzed similarly at least 91% of its activity remained if the enzyme was assayed before and after photolysis in the absence of cysteine; at least 97% of the activity remained if the enzyme was assayed in the presence of cysteine. The *p*-azidophenacyl group on the enzyme, then, may be selectively decomposed by photolysis with little, if any, damage to the enzyme, a result both of the fact that the inhibitor absorbs strongly above 300 nm and that the efficiency of nitrene formation from the excited state of the inhibitor is very high. (The quantum yield of loss of N_2 from *p*-azidoacetophenone upon photolysis at 280 or 365 nm is unity (Reiser and Marley, 1968).) We are presently engaged in determining the sites of insertion of the nitrene into GPDH.

We are also using **1** to probe the active sites of other enzymes which have groups that can selectively react with **1**: chymotrypsin (methionine), yeast alcohol dehydrogenase (cysteine), and pepsin (aspartic acid). Reagent **1** should be extremely useful with numerous enzymes and other biological macromolecules.

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