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Photoaffinity Labeling of Acyl-Coenzyme A:Glycine *N*-Acyltransferase with *p*-Azidobenzoyl-Coenzyme A†

Edward P. Lau, Boyd E. Haley, and Roland E. Barden*

ABSTRACT: A photolabile reagent, *p*-azidobenzoyl-CoA, has been synthesized and tested as a photoaffinity label for acyl-CoA:glycine *N*-acyltransferase (EC 2.3.1.13) from beef liver. *p*-Azidobenzoyl-CoA is an active-site-directed reagent for this *N*-acyltransferase, since it is an alternate substrate (K_m = 26 μM, when [glycine] = 100 mM). Ultraviolet irradiation of a mixture of *p*-azidobenzoyl-CoA and the *N*-acyltransferase produces irreversible inhibition. Benzoyl-CoA protects the enzyme from inhibition by photoactivated *p*-azidobenzoyl-CoA. Acyl-CoA:glycine *N*-acyltransferase is composed of a single polypeptide with a molecular weight of about 35 000. Photolabeling experiments show that there is one active site per molecule of enzyme.

Coenzyme A serves as a carrier of acyl groups in a large number of enzymatic reactions involved in such processes as the synthesis and oxidation of fatty acids, physiological acylations, and the oxidation of certain α-keto acids. Structural analogues of acyl-CoA capable of covalently labeling the coenzyme binding region of active sites (i.e., affinity labels) presumably would be a useful tool for characterizing structural and mechanistic aspects of these enzymes. Thus, the goal of our research is to develop analogues of acyl-CoA which meet the following basic criteria (see Shaw, 1970; Baker, 1967). (a) The analogue must form a reagent–enzyme complex by specifically binding at the active site. (b) The analogue must be capable of forming a covalent bond with a side chain located in the acyl-CoA binding region. In other words, the analogues are intended to be active-site-directed irreversible inhibitors for enzymes using acyl-CoA.

For our purposes, affinity labeling with a photogenerated reagent appeared to be a promising technique. Photoaffinity analogues can provide both specificity of interaction at the active site and the potential to generate a highly reactive intermediate capable of forming a covalent bond with any side chain in close proximity (Knowles, 1972). A suitable photogenerated group for affinity labeling is the arylnitrene system (e.g., Hixson and Hixson, 1975; Haley, 1975). Consequently, we have synthesized and characterized *p*-azidobenzoyl-CoA and have initiated testing this reagent as a photoaffinity label for enzymes using acyl-CoA. The present report describes studies with acyl-CoA:glycine *N*-acyltransferase (EC 2.3.1.13), an enzyme which synthesizes salicylurate (for excretion in the urine) from salicyl-CoA and glycine (Forman et al., 1971).

A preliminary report of some of these data has been presented (Lau and Barden, 1976).

Experimental Procedure

Materials. Key materials were obtained from commercial sources, as follows. CoA, Li^+ salt, was from P-L Biochemicals; *p*-aminobenzoic acid, *N*-hydroxysuccinimide, and dicyclohexylcarbodiimide were from Aldrich; *p*-amino[^{14}C]benzoic acid (40 Ci/mol) was from ICN; [^{3}H]CoA (500 Ci/mol) was from New England Nuclear; blue dextran, Sepharose 4B, and Sephadex G-100 were from Pharmacia; DEAE-cellulose was from Bio-Rad. The other chemicals used were of highest purity available from commercial sources.

Benzoyl-CoA was synthesized as described by Mieyal et al. (1974). *p*-Azidobenzoyl-CoA was prepared by acylating CoA with a 20% molar excess of the *N*-hydroxysuccinimide ester of *p*-azidobenzoic acid, as described in published procedures (Al-Arif and Blecher, 1969; Lapidot et al., 1967). *p*-Aminobenzoic acid was converted to *p*-azidobenzoic acid via a diazonium salt intermediate. *p*-Aminobenzoic acid (10 mmol) was suspended in 50 mL of 4 N sulfuric acid at 0 °C and dissolved by treatment with 12 mmol of sodium nitrite dissolved in 12 mL of water. After 10 min, 12 mmol of sodium azide in 12 mL of water was added. After stirring for 60 min, the precipitate was collected by filtration, washed thoroughly with cold water, and air dried (in the dark) (yield 80%; mp 180–181 °C (literature mp 180–182 °C; Merrill and Unruh, 1963)). Syntheses involving radioactive chemicals were also performed by the procedures outlined above, except that less materials were used so as to avoid excessive dilution of the radioactivity. Acyl-CoA preparations were routinely purified by chromatography on DEAE-cellulose column using a linear gradient of 0.08 to 0.3

† From the Department of Chemistry and Division of Biochemistry, University of Wyoming, Laramie, Wyoming 82071. *Received December 15, 1976.* This research was supported by a grant from the National Institutes of Health (GM 20077). R.E.B. is the recipient of a National Institutes of Health Research Career Development Award (GM 00246).

M LiCl followed by desalting on a Sephadex G-15 column (Fung, 1972).

The acyl-CoA:glycine *N*-acyltransferase used in this investigation was isolated from mitochondria of beef liver. A relatively crude fraction containing the *N*-acyltransferase activity was isolated from mitochondria following the procedure of Forman et al. (1971). This fraction (20 mL, 270 mg of protein, SA = 0.5) was dialyzed for 12 h against 3 L of 10 mM Tris-Cl, pH 8.0, with two changes of buffer. The dialyzed sample was further purified by affinity chromatography on a blue dextran–Sepharose 4B column (1.8 × 10 cm) (Ryan and Vestling, 1974) equilibrated with 10 mM Tris-Cl, pH 8.0. The glycine-dependent acyltransferase stuck to the column, and after washing with 100 mL of the original buffer the activity was eluted with 0.1 M NaCl in 10 mM Tris-Cl, pH 8.0. Fractions containing activity were pooled and concentrated to ~8 mL (19 mg of protein, specific activity 7.5 units/mg) with an Amicon microultrafiltration device, Model 8 MC. The concentrated enzyme solution was chromatographed on a Sephadex G-100 column (3.8 × 53 cm) equilibrated with 0.1 M Tris-Cl, pH 8.0. Fractions containing acyl-CoA:glycine *N*-acyltransferase were pooled (5.2 mg of protein; specific activity 12 units/mg), concentrated, and stored at −20 °C.

Methods. The purity of acyl-CoA preparations was assessed by thin-layer chromatography at room temperature on cellulose sheets containing a fluorescent indicator (Eastman Kodak, No. 13254); the solvent system was 1-butanol/glacial acetic acid/water (5:2:3, v/v). CoA compounds were located with a Mineralight UVS-11 lamp. The R_f of both *p*-azidobenzoyl- and benzoyl-CoA is ~0.59.

The extinction coefficient of *p*-azidobenzoyl-CoA was estimated by dissolving three carefully weighed samples in 0.1 M sodium phosphate, pH 7.0. A value of 22.2 ± 0.4 mM^{-1} at 265 nm was obtained. In these calculations, the weights of three Li^+ and three water of hydration were included in the formula weight of *p*-azidobenzoyl-CoA. Absorption spectra were measured on a Cary 14 spectrophotometer.

Acyl-CoA:glycine *N*-acyltransferase was assayed at 38 °C by detecting the glycine-dependent release of $CoAS^-$ with 5,5′-dithiobis(2-nitrobenzoic acid). The ΔA_{412} was measured with a Gilford recording spectrophotometer (Model 240) equipped with a constant-temperature circulating-water bath. Assay mixtures contained the following in a volume of 1.0 mL: 100 μmol of Na Hepes,[1] pH 8.0; 100 μmol of 5,5′-dithiobis(2-nitrobenzoic acid), 100 mmol of glycine, and benzoyl-CoA (100 nmol, unless noted otherwise). The reaction was initiated by addition of the enzyme. Hydrolysis of benzoyl-CoA in the absence of glycine was not detectable in the purified enzyme preparation. Enzyme units are expressed as μmoles of sulfhydryl group formed per minute at 38 °C.

Protein was determined by the method of Lowry et al. (1951) using crystalline bovine plasma albumin as the standard.

Electrophoresis on sodium dodecyl sulfate–polyacrylamide gels was performed using 10% acrylamide gels according to the procedure of Weber et al. (1972). Gels were stained with Coomassie brilliant blue for 2 h and destained at 20 °C for 48 h by the leaching method (Barden et al., 1975). Destained gels were scanned at 550 nm with the Gilford linear transport system. Radioactivity was detected in slices of polyacrylamide gel as previously described (Taylor et al., 1975).

The following proteins were used as standards for molecular weight determinations: hemoglobin, catalase, bovine serum albumin, alcohol dehydrogenase (yeast), malate dehydrogenase, citrate synthase (pig heart), and carnitine acetyltransferase (pigeon).

Photolysis of *p*-azidobenzoyl-CoA was performed with a Blak-Ray UVL-21 lamp (for which the wavelength of maximum intensity is 366 nm) positioned 2 cm from the sample, which was contained in a well of a Pyrex spot plate. Solutions were generally photolyzed for 5 min at 4 °C; mixing was achieved by magnetic stirring. In most experiments, two cycles of photolysis were performed; that is, after irradiation for 5 min, a second aliquot of *p*-azidobenzoyl-CoA was added and, after mixing, the system was photolyzed again for 5 min. In control experiments, it was shown that the *N*-acyltransferase per se is not inactivated by the photolysis procedure. Incorporation of radioactivity into protein was measured by the filter-paper disk method of Mans and Novelli (1961). Aliquots of an assay mixture were transferred onto Whatman 3MM paper disks, 2.3 cm in diameter. The disks were air dried and washed three times in ice-cold 10% trichloroacetic acid containing 10 mM AMP, and then washed, sequentially, with ethanol and diethyl ether. After drying the disks, protein-bound radioactivity was determined by placing each disk in 10 mL of scintillation cocktail (Aquasol 2, New England Nuclear) and counting with a Beckman liquid scintillation system, Model LS-250.

Results and Discussion

Molecular Size of Acyl-CoA:Glycine N-Acyltransferase from Beef Liver. A molecular weight of 36 000 was obtained for the *N*-acyltransferase by gel filtration on Sephadex G-100 (Andrews, 1965). Analysis of the purified *N*-acyltransferase by sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed two protein bands (Figure 1). The major component constituted ~90% of the sample and the minor component ~10%, as estimated from the areas under the peaks in scans such as that shown in Figure 1.[2] Molecular weight studies by the sodium dodecyl sulfate–polyacrylamide gel procedure (Weber et al., 1972) gave values of 34 000 for the major band and 31 000 for the minor band. The major component (Figure 1) appears to be the *N*-acyltransferase, since its molecular weight (34 000) correlates closely with that determined for the active enzyme (36 000). Furthermore, after photoaffinity labeling of the enzyme with *p*-azido[^{14}C]benzoyl-CoA and electrophoresis on sodium dodecyl sulfate–polyacrylamide gels, only the slice of gel containing the major component (34 000) exhibits levels of radioactivity significantly above background. A comparison of the molecular weights for the active enzyme (36 000) and for the constituent polypeptide chain (34 000) reveals that native *N*-acyltransferase is composed of a single polypeptide chain.

Photolability of p-Azidobenzoyl-CoA. Irradiation of *p*-azidobenzoyl-CoA should cause the photodecomposition of the azido group to the highly reactive nitrene, as described by Knowles (1972). The photolability of *p*-azidobenzoyl-CoA was demonstrated by two different methods. In the first method, a solution of *p*-azidobenzoyl-CoA was placed in a quartz cuvet and irradiated for set periods of time. At the end of each ex-

[1] Abbreviation used is: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

[2] In some preparations a third polypeptide was detected. Subsequent analysis of the fractions eluted from the Sephadex G-100 column showed that this polypeptide contaminated only the first few fractions containing the *N*-acyltransferase. Thus, disposal of the first portion of the *N*-acyltransferase peak will circumvent contamination by a third polypeptide.

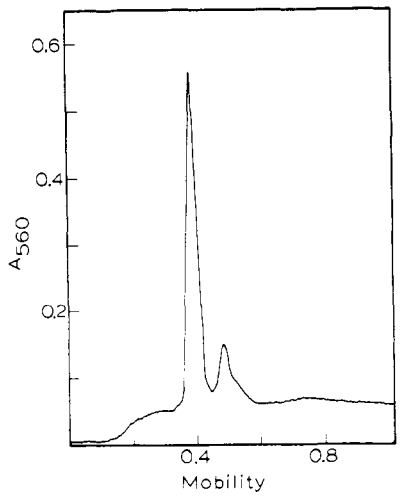

FIGURE 1: A spectrophotometric scan of a 10% polyacrylamide gel on which 8 μg of acyl-CoA:glycine *N*-acyltransferase (specific activity 12 units/mg) was analyzed by electrophoresis in the presence of sodium dodecyl sulfate. The protein was stained with Coomassie brilliant blue, and destained as described under Methods. The zero absorbance was set with the cuvet filled with water; thus, the apparent background absorbance is a consequence of the gel acting as a weak neutral filter to the incident light.

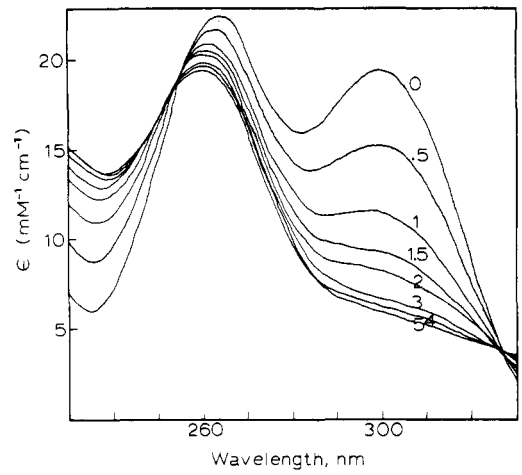

FIGURE 2: Absorption spectra of *p*-azidobenzoyl-CoA after irradiation for fixed lengths of time. Samples were dissolved in 0.1 M sodium phosphate, pH 7.0. Samples were photolyzed in a 3-mL quartz cuvet, and the lengths of exposure to irradiation (in minutes) are shown on the spectra.

posure to irradiation an absorption spectrum of the sample was obtained; several of these spectra are shown in Figure 2. As a consequence of irradiation, the intensity of the peak at 300 nm is greatly reduced and the peak at 265 nm undergoes a smaller decrease in intensity as its maximum shifts to 260 nm. Photolysis decreases the absorptivity at 265 nm from 22.2 to ~19 mM^{-1}. Photodecomposition of the sample is essentially complete after 4 min of irradiation (Figure 2).

In the second method, two spots of *p*-azidobenzoyl-CoA were placed at the origin on a cellulose sheet; one spot was irradiated with an ultraviolet lamp, the other was protected from the ultraviolet light. After development, examination of the plate with an ultraviolet lamp indicated that material from both spots migrated with an R_f of 0.59; however, a significant portion of the irradiated sample remained at the origin. Thus, a portion of the photoactivated sample became covalently bound to the cellulose and could not move.

Photolabeling of Acyl-CoA:Glycine N-Acyltransferase. Convincing evidence that *p*-azidobenzoyl-CoA is an active-site-directed reagent for acyl-CoA:glycine *N*-acyltransferase was observed in studies which demonstrated that the photolabile reagent is a good substrate for the *N*-acyltransferase. In the presence of 100 mM glycine, the following apparent kinetic constants were determined for *p*-azidobenzoyl-CoA: $K_m = 26 \pm 2$ μM, $V_{max} = 12 \pm 0.3$ nmol of -SH released per min. For comparison, a K_m of 37 ± 5 μM and V_{max} of 8.8 ± 0.7 nmol of -SH released per min was observed for benzoyl-CoA in the same experiment. (The kinetic constants were computed with the HYPERO program of Cleland (1967).) *p*-Azidobenzoyl-CoA thus has the same apparent affinity for the acyl CoA portion of the active site as benzoyl-CoA under these conditions. The observation that V_{max} is faster with *p*-azidobenzoyl-CoA than with benzoyl-CoA is an expected result, since the azido group is electron withdrawing and, consequently, the thioester carbonyl group of *p*-azidobenzoyl-CoA is more susceptible to nucleophilic attack than the thioester carbonyl group of benzoyl-CoA.

When the *N*-acyltransferase is incubated in the presence of *p*-azidobenzoyl-CoA, photolysis of the solution results in inhibition of the enzyme. An illustrative example of data from these experiments is shown in Table I. Concentrations of *p*-azidobenzoyl-CoA higher than 260 μM do not significantly increase the extent of inhibition. As expected, addition of benzoyl-CoA protects the *N*-acyltransferase from inhibition by photoactivated *p*-azidobenzoyl-CoA (Table I). We consistently observed that benzoyl-CoA is quite efficient at protecting the enzyme from photolabeling by *p*-azidobenzoyl-CoA.

TABLE I: Inhibition of Acyl-CoA:Glycine *N*-Acyltransferase with Photoactivated *p*-Azidobenzoyl-CoA[a]

p-Azidobenzoyl-CoA (μM)	Benzoyl-CoA (μM)	% Inhibition
43	0	4
85	0	10
128	0	17
256	0	31
128	110	9
128	164	0

[a] The irradiated mixture consisted of the following, in 150 μL: 13 μg of enzyme (~2 μM); 0.1 M Na Hepes, pH 8.0; 0.1 M KCl; the indicated concentrations of acyl-CoA. After irradiation for 5 min, activity was assessed by withdrawing 10-μL aliquots. Control experiments, in the presence and absence of benzoyl-CoA, show that the enzyme does not lose activity during the irradiation procedure.

Irreversible binding of photolyzed *p*-azidobenzoyl-CoA (labeled with either [^{3}H]CoA or *p*-azido[^{14}C]benzoic acid) to the *N*-acyltransferase was demonstrated by chromatographing a photolyzed reaction mixture on a 3.8 × 50 cm column of Sephadex G-100. The reaction mixture, which was photolyzed for 5 min, contained the following, in 2.0 mL: 0.78 mg of purified enzyme, 0.2 mmol of KCl, 0.2 mmol of Hepes, pH 8.0, and 18 nmol of *p*-azidobenzoyl-CoA. Analysis of the eluted fractions revealed that a peak of radioactivity coincided with the peak of enzyme activity regardless of whether the CoA portion or the *p*-azidobenzoyl portion of the reagent contained the radioactive label. Unbound reagent appeared at an elution volume which was twice that of the enzyme, and the two constituents were cleanly resolved. In addition to demonstrating

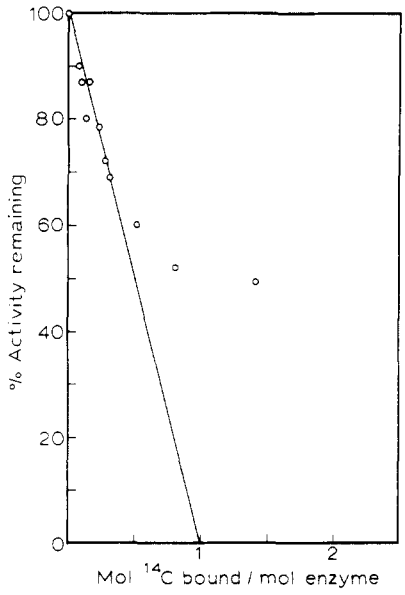

FIGURE 3: Determination of the number of active sites per molecule of acyl-CoA:glycine *N*-acyltransferase. The irradiated mixtures contained the following, in 150 μL: 13 μg of purified enzyme; 0.1 M KCl; 0.2 M Na Hepes, pH 8.0; and varying concentrations of *p*-azido[^{14}C]benzoyl-CoA (40–400 μM). After photolysis, duplicate 10-μL samples were withdrawn for enzyme activity assays, and duplicate 50-μL samples were withdrawn for measuring the moles of [^{14}C]reagent incorporated. Identical mixtures which were not photolyzed served as controls.

that the reagent is irreversibly bound to the inhibited enzyme, these studies show that the reagent is bound intact; i.e., loss of CoA through hydrolysis of the thioester linkage does not occur.

The number of active sites per molecule of *N*-acyltransferase was assessed with the photolabile reagent. In these experiments, a mixture of reagent and enzyme was photolyzed and samples were removed for measuring both activity and moles of [^{14}C]reagent bound, as described under Methods. The percent activity remaining was plotted vs. the moles of reagent bound per mole of enzyme, as shown in Figure 3. It was assumed that 90% of the protein present was *N*-acyltransferase (vide ante). An extended extrapolation of the straight line defined by the data at low levels of incorporation is required in order to intersect the abscissa (Figure 3). Nonetheless, the data clearly indicate that there is only one active site per molecule of *N*-acyltransferase.

Attempts were made to decrease the percent activity remaining to values less than ~65% by photolyzing the enzyme in the presence of relatively high concentrations of *p*-azidobenzoyl-CoA (>260 μM) or by repeated cycles of photolysis with "normal" concentrations of *p*-azidobenzoyl-CoA (50–250 μM). These procedures greatly increased the moles of reagent bound per mole of enzyme (presumably through nonspecific interactions) without significantly increasing the amount of inhibition achieved, as illustrated in Figure 3.

The stoichiometry of the number of active sites per mole of enzyme also was assessed by an alternate procedure. Affinity chromatography on a blue dextran–Sepharose 4B column was used to separate unmodified enzyme and the contaminant protein from the reagent–enzyme complex. In a typical experiment, 0.92 mg of enzyme and 137 μM *p*-azido[^{14}C]benzoyl-CoA were mixed, photolyzed, and chromatographed on the affinity column. Catalytic activity could not be detected in the reagent–enzyme complex eluting from the column. After the unbound reagent that contaminated the sample was removed by exhaustive dialysis, the moles of [^{14}C]reagent bound per mole enzyme was determined. A value of 0.9 ± 0.1 moles of reagent bound per mole of enzyme was obtained.

Concluding Comments. Affinity chromatography on a blue dextran–Sepharose 4B column has proved to be a useful purification procedure for a number of nucleotide-requiring enzymes (e.g., Thompson et al., 1975). Our studies show that this purification procedure is readily adapted to the isolation of acyl-CoA:glycine *N*-acyltransferase from beef liver and that a substantial gain in specific activity (~15-fold) is achieved by this operation. Similarly, a purification step based on complex formation between phenylacetyl-CoA:glutamine *N*-acyltransferase (monkey liver) and blue dextran was recently described by Webster et al. (1976).

A molecular weight of "approximately 32 000" for the glycine *N*-acyltransferase from beef liver was cited as unpublished data by Forman et al. (1971). In comparison, our studies indicate that the molecular weight of this enzyme is in the range of 34 000 (sodium dodecyl sulfate–polyacrylamide gel) to 36 000 (gel filtration). A molecular weight of 24 000 has been reported for the glycine *N*-acyltransferase from monkey liver (Webster et al., 1976).

It is tempting to extract apparent binding constants from the data in Table I by invoking simple models for reversible ligand binding. However, these data are not amenable to such analysis because of the complex nature of the photolabeling experiments. For instance, the concentration of the photoaffinity label changes rapidly during the 5 min of irradiation. In addition, most of the photodecomposition products are undoubtedly still structural analogues of an arylacyl-CoA (see Knowles, 1972), and, as such, they will compete with the photoaffinity label for the binding site. It is not possible to determine the extent of site protection afforded by the photodecomposition products. Obviously, the degree to which a given concentration of *p*-azidobenzoyl-CoA or benzoyl-CoA saturates the binding sites cannot be assessed unambiguously with this type of experiment. On the other hand, photoaffinity labeling experiments do answer certain key questions in a qualitative manner. Thus, the data in Table I clearly show that photolysis of a solution of enzyme and site-directed photogenerated reagent (*p*-azidobenzoyl-CoA) does produce irreversible inhibition; furthermore, addition of a second site-directed reagent (benzoyl-CoA) does produce the anticipated protection of the binding site.

Nonspecific binding of affinity labels through interaction with low affinity sites or through bimolecular collisions with protein can be a serious obstacle to both the determination of receptor site stoichiometry and the unambiguous labeling of an active-site residue(s) (Singer, 1970; Katzenellenbogen, 1974). Photoaffinity labels exhibit a particularly high chemical reactivity; thus, nonspecific incorporation is potentially a serious disadvantage of their use. However, our studies show that reliable active site stoichiometry can be obtained by careful studies using conditions under which moles of reagent bound per mole of enzyme is essentially proportional to percent activity remaining. A critical factor in an experiment of this type is to raise the concentration of receptor sites to a level near (or greater than, if practical) that of the photolabile reagent. This experimental situation leads to higher levels of incorporation, enhances the precision of the data, and extends (to a limited degree) the linear portion of the relationship between percent activity remaining and moles of reagent bound per mole of enzyme, as compared to systems in which the concentration of photolabile reagent far exceeds the concentration of available receptor sites.

A thorough kinetic analysis of acyl-CoA:glycine *N*-acyltransferase has not been reported, and, consequently, the reaction mechanism for this enzyme is not known. The studies described here seem to exclude one potential mechanism, however. Specifically, a double-displacement (i.e., ping-pong) mechanism involving a covalent acyl-enzyme intermediate would appear to be inconsistent with our observation that the photolabeled enzyme contains both the arylacyl and the CoA moieties of *p*-azidobenzoyl-CoA.

It is noteworthy that H. G. Wood and his co-workers have found *p*-azidobenzoyl-CoA to be a suitable photoaffinity label for methylmalonyl-CoA transcarboxylase (Poto and Lau, 1977). Conceivably, *p*-azidobenzoyl-CoA may be suitable for investigating receptor sites on other enzymes which utilize CoA esterified with an aliphatic acid.

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