THE 8-AZIDOADENINE ANALOG OF S-BENZOYL (3'-DEPHOSPHO)COENZYME A -- A PHOTOAFFINITY LABEL FOR ACYL CoA:GLYCINE N-ACYLTRANSFERASE

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Summary - S-benzoyl (3'-dephospho-8-azido)-CoA was synthesized by condensing 8-azido-AMP and S-benzoyl-4'-phosphopantetheine. The reagent was shown to be photosensitive. Studies with acyl CoA:glycine N-acyltransferase from beef liver show that the reagent is an alternate substrate and that photolysis of a mixture of reagent and enzyme causes irreversible inhibition. Thus, S-benzoyl (3'-dephospho-8-azido)-CoA is a photogenerated active-site-directed irreversible inhibitor (i.e. a photoaffinity label) for this N-acyltransferase.

Active-site-directed irreversible inhibitors (i.e. affinity labels) can be a useful tool for investigating enzyme chemistry (1,2). As pointed out by Knowles, irreversible inhibitors that are photogenerated offer certain advantages over reagents that bear more conventional functional groups (3). To date a number of photolabile reagents have been synthesized and used in photoaffinity labeling experiments (3,4). The synthesis of photolabile reagents that are analogs of coenzymes is especially attractive, because reagents of this type are potentially suitable for investigating the active site of every enzyme for which the particular coenzyme is a substrate. Noteworthy examples of this approach are found in the series of adenine nucleotides that have been made photolabile by derivatization at the C-8 position with an azido group. Thus the 8-azidoadenine analogs of 3',5'-cyclic AMP (5,6), ADP (7,8), ATP (9), and NAD^+ and FAD (10) have been synthesized and used in photoaffinity labeling experiments. We now report the synthesis of the 8-azidoadenine analog of (3'-dephospho)-Coenzyme A. When esterified

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with benzoic acid this reagent is a photoaffinity label for acyl CoA:glycine N-acyltransferase, a liver enzyme that synthesizes salicylurate.

Methods and Materials

Benzoyl CoA was synthesized as described by Mieyal et al (11). Acyl CoA:glycine N-acyltransferase (EC 2.3.1.13) was isolated from beef liver as previously described (12). The enzyme was approximately 90% pure on the basis of analysis by SDS-polyacrylamide gel electrophoresis (cf 12). Acyl CoA:glycine N-acyltransferase was assayed at 38° 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 equipped with a constant temperature circulating-water bath. Assay mixtures contained the following in a volume of 1.0 ml: 100 µmol sodium N-2-hydroxyethylpiperazine-N'-2-ethane sulfonate (HEPES), pH 8.0; 300 nmol 5,5'-dithiobis-(2-nitrobenzoic acid); 100 µmol glycine; variable concentrations of acyl CoA.

The synthesis of S-benzoyl (3'-dephospho-8-azido)-CoA was accomplished by coupling S-benzoyl-4'-phosphopantetheine to 8-azido-AMP using the anion exchange method of Michelson (13). A typical synthesis is described here. To prepare S-benzoyl-4'-phosphopantetheine, D-pantethine was first converted to D-pantethine-4',4"-diphosphate, calcium salt (14). To 12 µmol of this calcium salt in 1 ml of 0.5 M tris, pH 9.0, we added 90 µmol of $NaBH_4$, and the reaction mixture was incubated at 37° for 15 min. After cooling the mixture on an ice bath, the pH was adjusted to 7 with 2 M HCl. Benzoyl chloride (40 µl in 1.25 ml acetone) was added; after 20 min, benzoic acid and unreacted benzoyl chloride were extracted with diethyl ether (10 ml). The aqueous solution was chromatographed on a column (1.5 x 10 cm) of BioRad AG50W-X8 cation exchange resin in the hydrogen form. Fractions containing A_{232}-absorbing material were pooled, evaporated to dryness, and dissolved in dry dimethylformamide (5 ml).

The triethylammonium salt of 8-azido-AMP was synthesized and activated with diphenylchlorophosphate as previously described (6). The solution containing the S-benzoyl-4'-phosphopantetheine was combined with 10 ml of dimethylformamide containing 24 µmol of activated 8-azido-AMP and the system was diluted with 15 ml of dry pyridine. After 45 min, the sample was concentrated by rotary evaporation, and then diluted with H_2O prior to chromatography on a DEAE-cellulose column (1.9 x 22 cm). After washing with 100 ml of 0.08 M LiCl, a gradient of 0.08 to 0.3 M LiCl was initiated (cf 15). On the basis of TLC and UV absorption studies, a broad A_{260}-absorbing peak centered at 0.17 M LiCl was found to contain the product. The fractions were concentrated and chromatographed on a Sephadex LH-20 column (2.3 x 43 cm) equilibrated with ethanol:water (4:6) (cf 11) to remove a contaminant. The first peak of A_{260}-absorbing material to elute from this column appears to be P^1,P^2-di(8-azidoadenosine-5')diphosphate; the second peak is the desired product. The yield was 1 µmol; the product was stored frozen.

The purity of S-benzoyl (3'-dephospho-8-azido)-CoA was tested by thin layer chromatography on cellulose sheets (Eastman, #13254) with two different solvent systems,--butanol:acetic acid:H_2O (5:2:3), R_f = 0.63; ethyl alcohol: 0.5 N ammonium acetate, pH 3.8 (5:2), R_f = 0.81. Only one UV-absorbing spot was observed.

Results and Discussion

The Reagent is Photosensitive - When a sample of S-benzoyl (3'-dephospho-8-azido)-CoA is spotted at the origin on a cellulose sheet and irradiated with

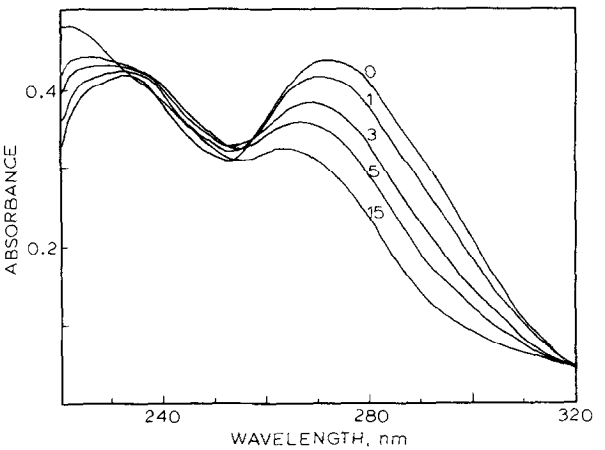

Figure 1. Absorption spectra of samples of S-benzoyl (3'-dephospho-8-azido)-CoA after irradiation for fixed lengths of time. The samples, dissolved in 0.25 M tris-Cl, pH 8, were contained in 3 ml quartz cuvets. The length of exposure to irradiation (in min) is shown on each of the spectra. The Mineral Light UVS lamp was set immediately adjacent to the cuvet.

a Mineral Light UVS lamp, a portion of the sample remains at the origin after development of the chromatogram. This observation indicates that upon photolysis the arylazide moiety decomposes to an arylnitrene, some of which becomes covalently attached to the cellulose.

The photosensitivity of the reagent was also demonstrated by a different experiment. Solutions of S-benzoyl (3'-dephospho-8-azido)-CoA were photolyzed for fixed periods of time, and after each exposure the UV spectrum was obtained. Several of these spectra are presented in Fig. 1. The absorption maximum at 272 nm exhibits a significant decrease in intensity upon photolysis, as expected for a photosensitive arylazide.

The Reagent is Active-Site-Directed - S-Benzoyl (3'-dephospho-8-azido)-CoA is a substrate for acyl CoA:glycine N-acyltransferase. In the presence of 100 mM glycine the apparent kinetic constants are: K_m = 13 μM and V_{max} = 7.2 nmol -SH formed per min. For comparison, the kinetic constants measured in the same experiment for benzoyl CoA are: K_m = 27 μM and V_{max} = 25 nmol

TABLE I

Inhibition of Acyl CoA:Glycine N-Acyltransferase with Photoactivated S-Benzoyl (3'-dephospho-8-azido)-CoA

S-Benzoyl (3'-dephospho-8-azido)-CoA, μM	Benzoyl CoA, μM	% Inhibition
6	-	10
12	-	17
36	-	24
70	-	26
140	-	33,32
140	140	20,22
140	280	0
280	-	38

The irradiated mixture, contained in the well of a spot plate, consisted of the following in 150 μl: 13 μg enzyme (∿2 μM); 0.1 M HEPES, pH 8.0; 0.1 M KCl; the indicated concentrations of acyl CoA. After irradiation for 5 min at 0° with a Mineral Light UVS lamp, activity was assayed by withdrawing 25 μl aliquots. Duplicate assays gave identical values, except where the results of both assays are shown. Control experiments showed that the N-acyltransferase does not loose activity when photolyzed for 5 min under these conditions.

-SH formed per min. These observations show that the photosensitive reagent binds reversibly at the active site.

The absorptivity of S-benzoyl (3'-dephospho-8-azido)-CoA was determined by measuring the amounts of thiol enzymatically released from samples of known A_{272}, as described by Webster, et al (16). At pH 8.0, ε_{272} is 20.3 mM^{-1}.

The Reagent is an Inhibitor - The data in Table 1 show that photolysis of a mixture of S-benzoyl (3'-dephospho-8-azido)-CoA and N-acyltransferase results in inhibition of the enzyme. Concentrations of the photolabile reagent in the range of its apparent K_m (see above) are effective, even

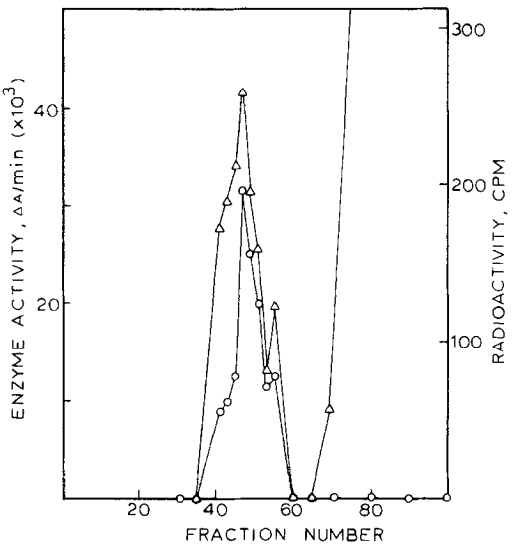

Figure 2. A demonstration that photolyzed S-benzoyl (3'-dephospho-8-azido)-CoA is irreversibly bound to the N-acyltransferase. The irradiated solution consisted of the following (in 3 ml): 700 µg enzyme; 0.1 M KCl; 50 mM HEPES, pH 8.0; 12 µM [^{14}C]-S-benzoyl (3'-dephospho-8-azido)-CoA. The sample was placed in a quartz cuvet, cooled to 0°, and photolyzed for 6 min with continuous stirring. The photolyzed solution was chromatographed at room temperature on a Sephadex G-100 column (3.8 x 53 cm) equilibrated with 0.1 M tris-Cl, pH 8.0. Fractions of 6.7 ml were collected, from which 0.1 ml aliquots were withdrawn for enzyme activity assays (O-O) and 1.0 ml aliquots for radioactivity assays (Δ-Δ).

though glycine is not present. Furthermore, in the concentration range tested an increase in the reagent level leads to an increase in the amount of inhibition achieved.

In a qualitative sense, one would expect that a second site-directed-reagent would compete with S-benzoyl (3'-dephospho-8-azido)-CoA for the binding site, and thus protect the enzyme from inhibition. As shown in Table 1, this phenomenon is observed. Addition of the alternate substrate, benzoyl CoA, to the reaction mixture protects the enzyme from inhibition by the photosensitive reagent.

The Reagent is Irreversibly Bound - To demonstrate that the inhibitor is irreversibly bound, a mixture of S-benzoyl (3'-dephospho-8-azido)-CoA (made

from [^{14}C]-AMP) and N-acyltransferase was photolyzed and then subjected to gel filtration chromatography on a Sephadex G-100 column. An analysis of the eluted fractions indicates that a peak of radioactivity coincides with the peak of enzyme activity (Fig. 2). Thus the reagent becomes irreversibly bound to the enzyme during the inhibitory process. Note that the peak of unbound reagent is cleanly resolved from the reagent-enzyme complex.

Concluding Comments - The evidence presented in this paper shows that S-benzoyl (3'-dephospho-8-azido)-CoA is a photogenerated active-site-directed irreversible inhibitor for acyl CoA:glycine N-acyltransferase. Presumably, analogous reagents suitable for studies with other acyl CoA-using enzymes can be prepared by esterifying the thiol with the appropriate acid.

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References

1. Baker, B. R. (1967) Design of Active-Site-Directed Irreversible Enzyme Inhibitors, John Wiley & Sons, New York.

2. Shaw, E. (1970) in The Enzymes (P. D. Boyer, Ed.), pp. 91-146, Academic Press, New York.

3. Knowles, J. R. (1972) Accts. Chem. Res., 5, 155-160.

4. Katzenellenbogen, J. A. (1974) Ann. Rep. Med. Chem., 9, 222-233.

5. Muneyama, K., Bauer, R. J., Shuman, D. A., Robins, R. K., and Simon, L. N. (1971) Biochemistry, 10, 2390-2395.

6. Haley, B. E. (1975) Biochemistry, 14, 3852-3857.

7. Schäfer, G., Schrader, E., Rowohl-Quisthoudt, G., Penades, S., and Rimpler, M. (1976) FEBS Lett., 64, 185-189.

8. Koberstein, R., Cobianchi, L., and Sund, H. (1976) FEBS Lett., 64, 176-180.

9. Haley, B., and Hoffman, J. F. (1974) Proc. Natl. Acad. Sci. (USA), 71, 3367-3371.

10. Koberstein, R. (1976) Eur. J. Biochem., 67, 223-229.

11. Mieyal, J. J., Webster, Jr., L. T., and Siddiqui, U. A. (1974) J. Biol. Chem., 249, 2633-2640.

12. Lau, E. P., Haley, B. E., and Barden, R. E. (1977) Biochemistry, (In Press).

13. Michelson, A. M. (1964) Biochim. Biophys. Acta, 91, 1-13.

14. Shimizu, M. (1970) Methods Enzymol., 18A, 322-338.

15. Fung, C.-H. (1972) Ph.D. Thesis, Case Western Reserve University.

16. Webster, Jr., L. T., Mieyal, J. J., and Siddiqui, U. A. (1974) J. Biol. Chem., 249, 2641-2645.