Photoaffinity Labeling of Acyl-CoA Oxidase with 12-Azidooleoyl-CoA and 12-[(4-Azidosalicyl)amino]dodecanoyl-CoA[†]

Ram Rajasekharan,[*] Russell C. Marians, Jay M. Shockey, and John D. Kemp

Plant Genetic Engineering Laboratory, New Mexico State University, Las Cruces, New Mexico 88003

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ABSTRACT: Synthesis of ^{32}P-labeled CoA of high specific activity was achieved using partially purified dephospho-CoA kinase (EC 2.7.1.24) from pig liver with [γ-^{32}P]ATP as donor and dephospho-CoA as acceptor. A photoaffinity dodecanoic acid analog, 12-[(4-azidosalicyl)amino]dodecanoic acid was synthesized, as were its CoA derivative (ASD-CoA) and the CoA derivative of 12-azidooleic acid. The CoA derivatives were synthesized from azido fatty acid analogs by acyl-CoA synthetase. The synthesized photolabile reagents were tested as photoaffinity labels for acyl-CoA oxidase (EC 1.3.99.3) from *Arthrobacter* species. When a mixture of oxidase and the acyl-CoA analogs were incubated in the absence of ultraviolet light, the analogs were recognized as substrate. Acyl-CoA oxidase was incubated in the presence of acyl-CoA analogs and immediately photolyzed, which resulted in irreversible inhibition. Oleoyl-CoA and dodecanoyl-CoA protect the enzyme from photoactivated inhibition by 12-azidooleoyl-CoA and ASD-CoA, respectively. Analysis of photolyzed enzyme preparations by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography revealed that both analogs preferentially labeled a 54 000 molecular weight protein. These results demonstrate that the photoaffinity acyl-CoA analogs have potential application as probes to identify and characterize lipid biosynthetic enzymes and to identify the active site of these proteins.

Fatty acyl-coenzyme A (acyl-CoA)[1] plays an important role in the metabolism of carbohydrates and fatty acids as an acyl group activator. Acyl-CoA is also a substrate for several enzymes that transfer fatty acids to proteins (Schultz et al., 1988, and references cited therein). Examples of proteins displaying acyltransferase activity are the growing numbers of N-acyltransferases and GTP-binding proteins found in both procaryotic and eucaryotic cells. Finally, acyl-CoA has also been deemed necessary for the budding of transport vesicles from Golgi cisternae (Pfanner et al., 1989). These observations suggest that acyl-CoA is directly involved in the biochemistry of bioenergetics and the regulation of enzyme activity through the posttranslational modification of proteins.

Acyl-CoA is a primary substrate for many membrane-associated proteins that catalyze lipid and wax biosynthesis in plants. Progress in the field of plant lipid metabolism has been hampered by the difficulty in purifying membrane-bound proteins, many of which are denatured upon solubilization. One alternative approach is the use of *Arabidopsis* mutants defective in lipid metabolism (Somerville & Browse, 1991). A second approach is the use of photoactive acyl-CoA to detect and label specific acyl-CoA binding proteins. In the course of our work studying oil and wax biosynthetic pathways in oilseeds, we have synthesized photoaffinity radiolabeled acyl-CoA of high specific activity. The azido analogs of fatty acids (Chakrabarti & Khorana, 1975) and of the CoA thioesters of fatty acids (Guillory & Jeng, 1977; Barden et al., 1983; Kinnunen et al., 1990) have proven to be very effective photoprobes for a number of proteins.

In the present study, we describe a simple enzymatic synthesis of CoA, 12-azidooleoyl-CoA, oleoyl-CoA, 12-[(4-azidosalicyl)amino]dodecanoyl-CoA (ASD-CoA), and their radiolabeled derivatives. To study the efficacy of the synthesized analogs of acyl-CoA, acyl-CoA oxidase from *Arthrobacter* was targeted for specific covalent labeling. The analogs were efficiently used as substrates in the dark, indicating that they bind to the acyl-CoA binding site. Upon irradiation in the presence of the photoprobes, oxidase activity was irreversibly inhibited, and the inhibition was diminished by the addition of oleoyl-CoA or dodecanoyl-CoA. These experiments suggest that 12-azidooleoyl-CoA and ASD-CoA act as active site specific photoaffinity labels for acyl-CoA oxidase.

EXPERIMENTAL PROCEDURES

Materials. [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from E. I. DuPont de Nemours Co. (Boston, MA). Ammonium persulfate and reagents for electrophoresis were obtained from Bio-Rad, Richmond, CA. N-Hydroxysuccinimidyl-4-azidosalicylic acid was obtained from Pierce, Rockford, IL. All other chemicals and enzymes were purchased from Sigma Chemical Co. (St. Louis, MO). Fresh pig liver was obtained from a local farm and stored at –70 °C until use.

Assay for Dephospho-CoA Kinase Activity. The enzyme was assayed for the production of ADP from dephospho-CoA and ATP by coupling with pyruvate kinase and lactate dehydrogenase by the method of Jaworek and Welsch (1985). The assay mixture consisted of 50 mM Tris-HCl (pH 8.0), 0.2 mM dephospho-CoA, 2 mM ATP, 3 mM MgCl$_2$, 0.2 mM NADH, 3 mM phosphoenolpyruvate, 5 units of pyruvate kinase (EC 2.7.1.40), 5 units of lactate dehydrogenase (EC 1.1.1.27), and the appropriate amount of enzyme in a total volume of 0.5 mL. The incubation was performed at 28 °C for 20 min and was stopped by boiling the tubes for 2 min, followed by centrifugation for 5 min to remove the proteins. The change in absorbance at 340 nm was monitored.

* To whom correspondence should be addressed.
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[1] Abbreviations: ASD-CoA, 12-[(4-azidosalicyl)amino]dodecanoyl-CoA; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Acyl-CoA Synthetase Assay. The reaction mixture consisted of 50 mM Tris-HCl (pH 8.0), 0.5 mM CoA (0.5 mCi/mmol), 5 mM ATP, 7.5 mM MgCl$_2$, 1 mM fatty acid, and 0.3% Triton X-100, in a total volume of 50 μL. The mixture was sonicated, and 50 μL acyl-CoA synthetase (0.1 unit) was added. The reaction was carried out at 28 °C for 20 min, then boiled for 2 min, and centrifuged. An aliquot of the reaction mixture was chromatographed on a silica gel thin-layer plate using a mixture of 1-butanol, acetic acid, and water (5:2:3, v/v), and the area corresponding to acyl-CoA was scraped and counted.

Acyl-CoA Oxidase Assay. Acyl-CoA oxidase was purchased from Sigma and used without purification. The reaction mixture contained 100 mM Tris-HCl, pH 8, 0.1 mM FAD, 0.1 mM acyl-CoA, 0.5 unit of oxidase, and 0.2 mL of a mixture of Ti(IV) and 4-(2-pyridylazo)resorcinol in a total volume of 0.5 mL. The incubation was carried out for 10 min at 30 °C. After incubation, the solution was cooled to room temperature, and the absorbance was measured at 508 nm (Matsubara et al. 1983).

Purification of Dephospho-CoA Kinase. Purification was carried out as previously described (Worrall & Tubbs, 1983) with minor modifications. Unless stated otherwise, all operations were conducted at 4 °C. Buffer A contained 20 mM Tris-HCl (pH 8.0), 0.5 mM DTT, 0.1 mM EDTA, 0.2 mM PMSF, and 0.25 M sucrose; buffer B contained 10 mM Tris-HCl (pH 8.0), 0.5 mM DTT, and 0.1 mM EDTA. Frozen pig liver (100 g) was ground in a Waring blender with 2 parts (w/v) of buffer A. The extract was centrifuged at 25000g for 30 min. The supernatant was further centrifuged at 100000g for 90 min to remove the particulate fraction. To the resulting supernatant, 2% protamine sulfate was slowly added to a final concentration of 0.1%. The suspension was stirred for 10 min and then centrifuged at 25000g for 30 min. The pellet was discarded, and the supernatant was brought to 50% saturation by the addition of solid ammonium sulfate. The solution was stirred for 30 min and centrifuged as above. The pellet was suspended in 25 mL of buffer B and dialyzed extensively against the same buffer.

The dialyzate was clarified by centrifugation and then loaded onto a Procion red-Sepharose column (4.4 × 10 cm) that had been equilibrated with buffer B. The column was washed with the same buffer and then eluted with 50 mL of buffer B that contained 0.6 M KCl. The eluted enzyme was dialyzed against buffer B. Dialyzed enzyme was loaded at a flow rate of 1 mL/min onto a blue-Sepharose (4.4 × 7.5 cm) column which had been equilibrated with buffer B. The column was washed with the same buffer until the effluent had a very low absorbance at 280 nm. The column was then washed with 50 mL of buffer B that contained 0.1 M KCl. The enzyme was eluted with buffer B containing 0.1 mM CoA and 0.1 M KCl. Fractions of 2 mL each were collected, and the active fractions were pooled, dialyzed against buffer B, and stored at –70 °C.

Preparation of Dephospho-CoA. Dephospho-CoA and acyl-dephospho-CoA were prepared by treating CoA and acyl-CoA with nuclease P1 (EC 3.1.30.1). The reaction mixture, in a total volume of 100 μL, contained 50 mM Tris-HCl (pH 8.0) 1 mM DTT, 2 mM substrate, and 100 μg of enzyme. The components were mixed and incubated at 37 °C for 30 min. The reaction was stopped by boiling the assay mixture for 2 min, followed by centrifugation at 4 °C for 5 min. An aliquot of oleoyl-dephospho-CoA preparation was chromatographed on a silica gel thin-layer plate using a mixture of 1-butanol, acetic acid, and water (5:2:3, v/v) and scraped to count the unreacted acyl-CoA. Acyl-dephospho-CoA was

also prepared chemically via the acid chloride of oleic acid as previously described (Bishop & Hajra, 1980).

Enzymatic Synthesis of ^{32}*P-Labeled CoA.* CoA was synthesized by scaling up the dephospho-CoA kinase reaction. The assay mixture contained 50 mM Tris-HCl, pH 8.0, 0.2 mM dephospho-CoA, 10 μM [γ-^{32}P]ATP ($\sim$10 Ci/mmol), 0.5 mM MgCl$_2$, 0.5 mM β-mercaptoethanol, and 0.5 unit of dephospho-CoA kinase in a total volume of 0.5 mL. The reaction was incubated for 90 min at 37 °C. CoA was separated from the other reaction products by DEAE-Sephadex (Pharmacia, A-25, Cl$^-$ form) column chromatography (Mukai et al., 1983). The reaction mixture was applied onto the column (1 × 20 cm) which had been previously washed and equilibrated with 50 mM Tris-HCl, pH 8.0, and 10 mM β-mercaptoethanol. The column was washed with the same buffer, and the labeled CoA was eluted with a step gradient (50, 100, 200, and 300 mM) of LiCl in buffer. One milliliter fractions were collected in microfuge tubes. The radioactive fractions containing CoA, which eluted at 300 mM LiCl, were pooled, concentrated by speed-vac, and stored at –20 °C. The purity of the product was checked on cellulose thin-layer plate as described by Mukai et al. (1983). The concentration of CoA was determined by the catalytic method with phospho-acetyltransferase (Michal & Bergmeyer, 1985).

Chemical Synthesis of 12-[(4-Azidosalicyl)amino]dodecanoic Acid. 12-[(4-Azidosalicyl)amino]dodecanoic acid was synthesized by dissolving 32 mg of 12-aminododecanoic acid and 26 mg of N-hydroxysuccinimide ester of 4-azidosalicylic acid in 2 mL of anhydrous THF and 0.2 mL of pyridine (Kinnunen et al., 1990). The reaction was stirred for 72 h in the dark at room temperature. Concentrated HCl (0.2 mL) was added to the mixture, which was then evaporated to dryness under vacuum. The residue was dissolved in 10 mL of ethyl acetate and extracted twice with 5 mL of 30 mM HCl. The organic phase was dried over sodium sulfate and then concentrated by evaporation. The reaction product was purified by preparative silica gel thin-layer chromatography using chloroform/methanol (5:1, v/v) as the solvent system followed by extraction from the silica gel. The purified photoreactive product migrated as a single spot. The R_f values for 12-aminododecanoic acid and 12-[(4-azidosalicyl)amino]dodecanoic acid were 0.00 and 0.63, respectively.

Synthesis of Acyl-CoAs. The reaction mixture consisted of 50 mM Tris-HCl (pH 8.0), 2 mM CoA, 5 mM ATP, 7.5 mM MgCl$_2$, 1 mM oleic acid or 12-aminododecanoic acid, and 0.3% Triton X-100. The mixture was sonicated for 5 min to emulsify the fatty acid, and then acyl-CoA synthetase (0.2 unit) was added to give a final volume of 0.7 mL. The reaction was carried out at 28 °C for 2 h. After incubation, the reaction was boiled for 2 min and centrifuged. Acyl-CoA was purified by the method of Molaparast-Saless et al. (1988), with minor modifications. The supernatant was chromatographed onto a 1-mL disposable Prep-Sep C18 reverse-phase extraction column (Fisher Scientific, Fair Lawn, NJ) which had been washed with 3 mL of methanol and equilibrated with 50 mM Tris-HCl, pH 8.0. After the sample was loaded, the column was washed with 1 column volume of buffer followed by 1 column volume of 50% methanol. Acyl-CoA was eluted with 5 mL of methanol, and the solvent was evaporated to dryness. The residue was redissolved in a small volume of methanol and stored at –20 °C. The synthesis of 12-azidooleoyl-CoA and ASD-CoA was carried out using identical methods as described above; however, all operations were carried out in the dark.

Table I: Purification of Dephospho-CoA Kinase from Pig Liver

steps	volume (mL)	protein (mg)	unit/mg (nmol)	total activity (μmol/min)	yield (%)	purification
100000g supernatant	175	15 510	0.31	4.81	100	1
protamine sulfate	190	8 320	0.53	4.41	92	1.7
ammonium sulfate	53	1 846	1.70	3.14	65	5.5
red-Sepharose	35	43	61.54	2.65	55	199
blue-Sepharose	12	1.23	1589	1.96	41	5126

Table II: Kinetic Results of Acyl-CoA Synthetase with Azido Fatty Acids[a]

substrate	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})
oleic acid	96	2.62
12-azidooleic acid	98	2.23
dodecanoic acid	116	1.74
azidosalicyldodecanoic acid	134	2.08

[a] Enzyme activity was measured by the formation of acyl-[^{32}P]CoA. The constant for fatty acids and the analogs (varied between concentrations of 5 and 400 μM) were derived with FAD maintained at concentrations of 0.2 mM. Apparent K_m and V_{max} values were determined from Lineweaver–Burk plots.

Photoaffinity Labeling. Photolabeling reactions were carried out on ice in the dark for 10 min before exposure with a hand-held UV lamp with the filter removed (5000 μW/cm^2, Model UVG-54, Ultraviolet Products, Inc., San Gabriel, CA) at a distance of 8 cm for 2 min. The photolabeling reaction was carried out in the cap of a microcentrifuge tube in a final volume of 25 μL containing 8 μg of enzyme in 25 mM Tris-HCl, pH 8.0, and 0.25 μCi (2 μM) of the labeled photoprobe. As a control, the substrate was first exposed to UV and then added to the enzyme. The labeling reaction was quenched by the addition of an equal volume of the protein-solubilizing mixture, which consisted of 0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, and 0.002% bromophenol blue. The samples were directly analyzed by SDS–PAGE (12%) using a standard Tris–glycine system (Laemmli, 1970). The gel was stained for protein using Coomassie blue R-250 and dried, and labeled bands were identified by autoradiography. These experiments were repeated three times, each from different preparations of azidoacyl-CoA. The stained protein bands were excised, and radioactivity was determined by liquid scintillation counting.

Protein Determination. Protein concentrations were assayed by the bicinchoninic acid method (Smith et al., 1985) using bovine serum albumin as the standard.

RESULTS

Purification of Dephospho-CoA Kinase. Table I summarizes the purification of dephospho-CoA kinase from pig liver. The enzyme was localized in the cytosol. Since the soluble fraction contained the highest amount of activity, attempts were made to purify the enzyme from this fraction. The enzyme activity was quite stable in a 100000g supernatant and could be stored as such at 4 °C for 3 days, while retaining up to 80% of the activity. The protamine sulfate and ammonium sulfate precipitations resulted in a 5-fold increase in specific activity and an overall recovery of 65%. The red-Sepharose chromatography step (batch elution) resulted in a 36-fold purification. At this stage a significant amount of phosphatase activity remained, so the kinase was further purified by blue-Sepharose chromatography and CoA affinity elution, producing an additional 26-fold purification. The overall recovery of the purified kinase was 41%; it had a specific activity of 1.96 μmol min^{-1} mg^{-1} and was phosphatase-free. The enzyme was stable at –70 °C for a minimum of 6 months.

Synthesis of ^{32}P-Labeled CoA. The synthesis of ^{32}P-labeled CoA was accomplished by the synthesis of dephospho-CoA from CoA by nuclease P1 followed by the conversion of the product back to CoA using a partially purified dephospho-CoA kinase preparation and [γ-^{32}P]ATP. Dephospho-CoA was obtained from CoA in the yield of 96% using a commercial preparation of nuclease P1. The enzyme also hydrolyzed acyl-CoA and aminododecanoyl-CoA with 85–90% efficiency. The resulting dephospho-CoA preparation was used in the kinase

reaction without purification. This material was converted to [^{32}P]CoA using our kinase preparation (see Experimental Procedures). The radioactively labeled substrate and product were separated by DEAE column chromatography using a LiCl step gradient. The ATP eluted at 100–150 mM, and the CoA eluted at 300 mM LiCl. The final purified product was thin-layer chromatographed and found to be pure with an R_f of 0.23. The yields of [^{32}P]CoA were in the range of 52–57%, based on the amount of [^{32}P]ATP used. The specific activity of the synthesized CoA was ~10 Ci/mmol.

Synthesis of Acyl-CoAs. Acyl-CoA was synthesized by coupling the dephospho-CoA kinase product with fatty acids or fatty acid analogs using commercially available acyl-CoA synthetase. The acylated product was purified by binding to a C18 reverse-phase column followed by elution with absolute methanol. The purity of the resulting product was assessed by silica gel TLC using 1-butanol:acetic acid:H$_2$O (5:2:3, v/v) as the solvent. A single radioactive and iodine-positive spot was observed with the mobility expected for authentic oleoyl-CoA (R_f = 0.51). No free CoA, fatty acid, or ATP was detected. The purified acyl-CoA contained <8% of acyl-dephospho-CoA (R_f = 0.53). Earlier results had demonstrated that the synthetase also accepts dephospho-CoA as substrate, although at a considerably lower rate than with CoA (Tubbs & Garland, 1969). This procedure has been used to prepare 12-azidooleoyl- and ASD-CoAs, in final yields of 41 and 33%, respectively, based on the amount of ATP used. 12-Azidooleoyl-CoA and ASD-CoA each had an R_f of 0.52. The ratios of absorbance at 232 versus 260 nm were 0.56 and 0.49 for azidooleoyl- and ASD-CoAs, respectively.

Table II compares the kinetics of acyl-CoA synthetase for fatty acids and their analogs. The K_m and V_{max} values for azidooleic acid are the same as those obtained with oleic acid. The K_m values for dodecanoic acid and azidosalicyldodecanoic acid were 116 and 134 μM, respectively. Acyl-CoA synthetase from *Pseudomonas* uses both short- and long-chain fatty acids as well as their azido analogs. However, the same enzyme from rat liver did not use a fluorescent fatty acid analog 16-(9-anthroyloxy)palmitic acid as substrate for acylation (Merrill et al., 1983). This suggests that the enzyme from *Pseudomonas* has broader substrate specificity than the enzyme from rat liver.

The extensively dialyzed commercial acyl-CoA oxidase was subjected to ultraviolet-visible absorption spectrum. The enzyme showed three major absorbtion maxima at 276, 364, and 447 nm, which are characteristics of a flavoprotein (data not shown). The specific activity of the dialyzed enzyme was low (18.74 μmol min^{-1} mg $^{-1}$) without the exogenous addition of FAD using dodecanoyl-CoA as a substrate. Exogenous FAD was required to obtain high specific activity (56.24 μmol min^{-1} mg^{-1}). FMN was ineffective in replacing FAD for obtaining the maximal velocity of the reaction. The biological activity of the synthesized acyl-CoA analogs was

Table III: Kinetic Constant for Acyl-CoA Oxidase[a]

substrate	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})
dodecanoyl-CoA	42	58
ASD-CoA	47	53
oleoyl-CoA	29	38
12-azidooleoyl-CoA	43	17

[a] Enzyme activity was measured by the formation of H_2O_2 with various concentrations of fatty acyl-CoAs (varied between concentrations of 1 and 250 μM). Apparent K_m and V_{max} values given were derived from Lineweaver–Burk type plots for acyl-CoA oxidase.

tested by their ability to function as substrates for acyl-CoA oxidase. In the presence of 0.2 mM FAD, the apparent kinetic constants were determined by use of dodecanoyl-, ASD-, oleoyl-, and azidooleoyl-CoA (Table III). The maximum velocities and Michaelis constants for dodecanoyl-CoA and ASD-CoA were about 1.5-fold higher than for oleoyl-CoA. The V_{max} for 12-azidooleoyl-CoA was 3.4-fold lower than that for the short-chain acyl-CoA and its analog. These results suggest that oleoyl-CoA is a better substrate for the oxidase from *Arthrobacter*. In contrast, the enzyme from *Candida tropicalis* used dodecanoyl-CoA as a better substrate than oleoyl-CoA (Shimizu et al., 1979a). We have also demonstrated that the photolabile reagents are good substrates in the dark, implying that these analogs bind to the active site of the enzyme.

Photolabeling of Acyl-CoA Oxidase. To demonstrate that azidooleoyl- and ASD-CoAs were binding to the active site of oxidase rather than nonspecifically to the protein, the holoenzyme was photolyzed with 2 μM [^{32}P]-12-azidooleoyl-CoA and [^{32}P]-ASD-CoA in the presence of FAD and analyzed by SDS–PAGE and autoradiography. The SDS–PAGE pattern of the photolyzed enzyme is shown in Figure 1. The presence of radioactivity exclusively in the gel region containing the 54-kDa polypeptide confirms the evidence obtained from kinetic data that the active site of this enzyme is specifically labeled upon photolysis in the presence of azidooleoyl- and ASD-CoAs. To further demonstrate the specificity of labeling, we have dialyzed broad-range molecular weight marker proteins (Bio-Rad) to remove DTT in the samples and then photolyzed them in the presence of azidoacyl-CoAs. Figure 2 shows that bovine serum albumin (66.2-kDa) was specifically labeled by both the azidoacyl-CoAs. It has been shown that albumin binds to acyl-CoA in an equimolar ratio (Perez-Gil et al., 1990). These experiments provide additional evidence for the specificity of the synthesized photoprobes.

To further demonstrate the ability of 12-azidooleoyl- and ASD-CoAs to specifically inhibit acyl-CoA oxidase activity upon irradiation, a series of different conditions were studied (Table IV). Acyl-CoA oxidase activity was unaffected by UV light in the presence of either oleoyl-CoA or dodecanoyl-CoA. In the presence of 100 μM 12-azidooleoyl-CoA or ASD-CoA, 51–58% of the activity was lost after 2 min of photolabeling. These results strongly suggest that the analogs were photoinserted into the acyl-CoA binding site on the enzyme in a specific manner.

When the oxidase was photolabeled with 5 μM of photoprobes in the presence of an increasing concentration of acyl-CoAs, approximately 70% and 63% of the labeling was protected by 25 μM oleoyl- and dodecanoyl-CoAs, respectively (Figure 3). The half-maximal protection of photolabeling was observed with 12–14 μM of acyl-CoA. These results suggest that the probes are photolabeling the active site of the enzyme.
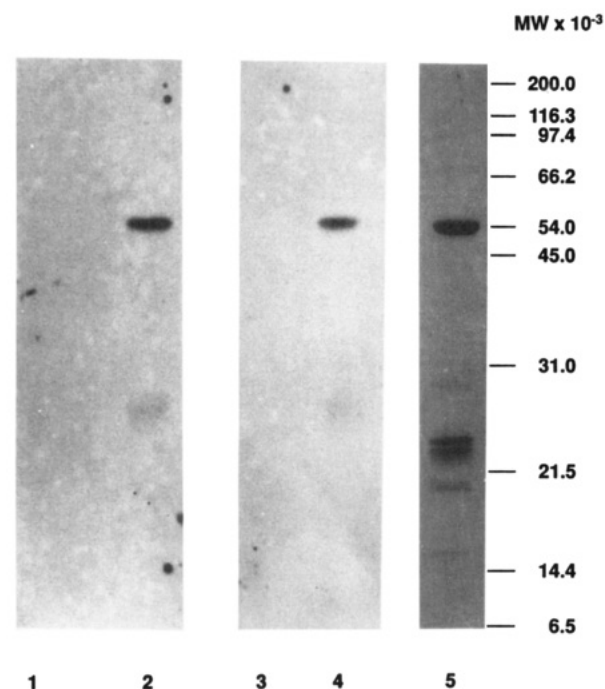
MW x 10^{-3}

FIGURE 1: SDS–PAGE and autoradiography of acyl-CoA oxidase photolabeled with [^{32}P]acyl-CoA analogs. Lanes 1–4 each contained 8 μg of enzyme and 0.25 μCi of the appropriate acyl-CoA analog. Azidooleoyl-CoA (lane 1) and ASD-CoA (lane 3) were first exposed to UV and then added to the enzyme. In lanes 2 and 4, azidooleoyl-CoA and ASD-CoA, respectively, were photolyzed in the presence of the enzyme. Lane 5 represents the Coomassie blue stain of acyl-CoA oxidase. Positions of molecular weight markers are indicated to the right.

DISCUSSION

In this paper, we describe an efficient enzymatic synthesis of [^{32}P]-labeled CoA. The synthesis uses a partially purified dephospho-CoA kinase which phosphorylates the 3′-position of dephospho-CoA. Neither this enzyme nor radiolabeled CoA are commercially available. This enzyme has been isolated from various sources; we purified our enzyme from pig liver because it was more stable and a well-defined purification procedure was available (Worrall & Tubbs, 1983). The overall yield of the kinase purification was 41%. Both blue-Sepharose and red-Sepharose were used in the purification. Red-Sepharose/salt elution gave the highest increase in specific activity, but blue-sepharose/CoA elution was needed to remove residual phosphatase activity. Dephospho-CoA was labeled with efficiencies of 60% and 18% with and without the blue-Sepharose step, respectively. The enzyme did not phosphorylate acylated dephospho-CoA, indicating that the phosphorylation is highly specific. The labeled CoA is obtained in high yield (>50%) with high specific activity (~10 Ci/mmol). Previous methods for the preparation of labeled CoA have been unsatisfactory because they require a large amount of radiolabeled substrate while returning only a small amount of product with a low specific activity of 9000 cpm/mmol (Shimizu et al., 1979b). Recently, dephospho-CoA kinase from *Brevibacterium ammoniagenes* has been successfully used to synthesize CoA and its analogs (Martin & Drueckhammer, 1992).

Specific hydrolysis of the 3′-phosphate of CoA by nuclease P1 allows a simple and rapid preparation of dephospho-CoA. It should be noted that the molecular and enzymatic properties of nuclease P1 are well-understood (Fujimoto et al., 1974) and that homogeneous enzyme preparations are available from many commercial sources. This enzyme also dephosphorylates
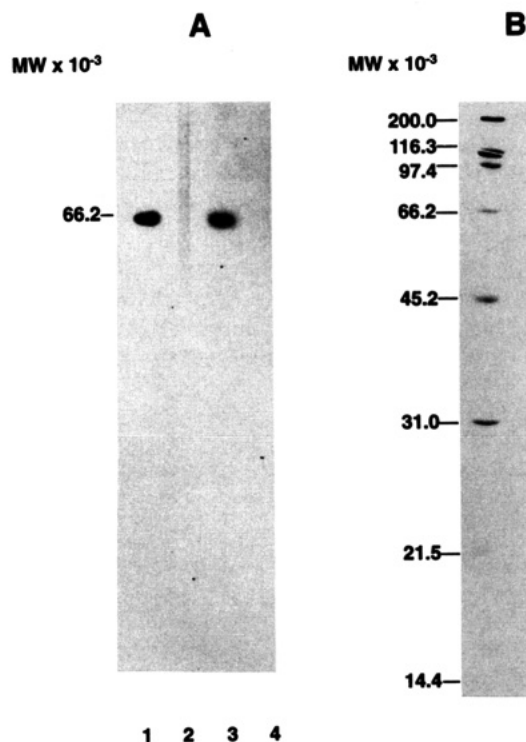
A

MW x 10⁻³

B

MW x 10⁻³

200.0—
116.3—
97.4—

66.2—

45.2—

31.0—

21.5—

14.4—

1 2 3 4

FIGURE 2: SDS–PAGE and autoradiography of broad range molecular weight markers (Bio-Rad) photolabeled with [³²P]acyl-CoA analogs. Lanes 1–4 each contained 13 μg of proteins and 0.25 μCi of the appropriate acyl-CoA analog (A). In lanes 2 and 4, azidooleoyl-CoA and ASD-CoA, respectively, were first exposed to UV and then added to the marker proteins. In lanes 1 and 3, azidooleoyl- and ASD-CoAs were photolyzed in the presence of proteins, respectively. Panel B represents the Coomassie blue stain of molecular weight marker proteins.

FIGURE 3: Protection against photolabeling of acyl-CoA oxidase by [³²P]-12-azidooleoyl- and ASD-CoAs in the presence of an increasing concentrations of oleoyl-CoA (●) and dodecanoyl-CoA (O), respectively. The photolabeling conditions and analysis are described under Experimental Procedures. The concentration of the photoprobe used was 5 μM. Values are expressed as mean cpm of two determinations.

Table IV: Photoinactivation of Acyl-CoA Oxidase by Azidoacyl-CoA Analogs

addition	UV irradiation	activity (%)
100 μM oleoyl-CoA	+	100
100 μM azidooleoyl-CoA	–	97
100 μM azidooleoyl-CoA	+	58
100 μM azidooleoyl-CoA + 1 mM oleoyl-CoA	+	89
100 μM dodecanoyl-CoA	+	100
100 μM ASD-CoA	–	95
100 μM ASD-CoA	+	51
100 μM ASD-CoA + 1 mM dodecanoyl-CoA	+	86

a The 0.1 mL of enzyme (8 μg) was photolyzed in 50 mM Tris-HCl, pH 8.0, containing 100 μM FAD and 100 μM azidoacyl-CoA analogs. An aliquot (25 μL) was taken and assayed as described under Experimental Procedures. Photolysis was performed by placing the samples on ice and irradiating them with an ultraviolet lamp at a distance of 8 cm between the lamp and the sample. The presence or absence of UV light prior to assay is indicated by + or –, respectively. Values are expressed as the mean of three determinations.

the 2′-position of NADP⁺ but does not dephosphorylate the 5′-position of AMP or ATP (data not shown).

We have synthesized radiolabeled CoA by the phosphorylation of dephospho-CoA with dephospho-CoA kinase. The radiolabeled CoA was linked to a fatty acid or its azido analogs to form acyl-CoA using the commercially available acyl-CoA synthetase from *Pseudomonas* spp. This enzyme has been used successfully for the synthesis of 12-azidooleoyl- and ASD-CoAs. This suggests that the enzyme has broad substrate specificity (Table II). Previous studies have shown that this enzyme activates saturated, unsaturated, and hydroxylated fatty acids with various carbon chain length in yields of greater than 90% (Taylor et al., 1990). In contrast, the efficiency of
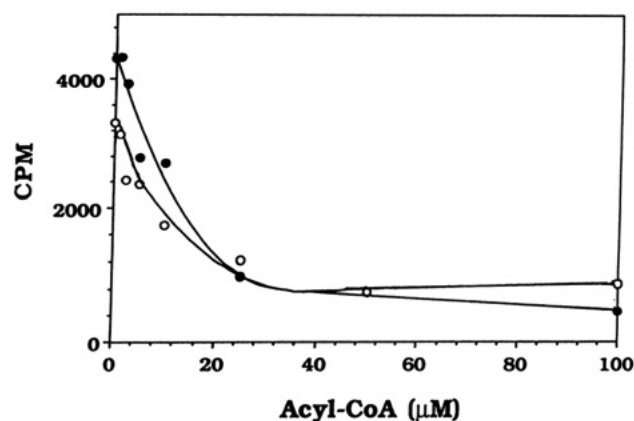
coupling fatty acid analogs to CoA in our hands was only 60%, which may be due to the low concentration of thiol reagent used (0.4 mM β-mercaptoethanol). We could not increase the thiol concentration to achieve higher yields because of the instability of the azido group (Cartwright et al., 1976).

The synthesized acyl-CoA analogs fulfill all the requirements necessary of a photoaffinity probe to be used in the study of acyl-CoA binding proteins. Acyl-CoA oxidase uses acyl-CoA analogs as a substrate in the dark, which confirms interaction at the active site of the enzyme. Photolysis of labeled 12-azidooleoyl- and ASD-CoAs prior to the addition of the enzyme did not lead to labeling of protein, eliminating the possibility of the existence of any long-lived chemically reactive intermediates which could be involved in covalently modifying the enzyme. Acyl-CoA prevents photoinsertion of [³²P]azidoacyl-CoA (Table IV and Figure 3), clearly demonstrating that the photoprobes are interacting with acyl-CoA binding site(s).

Among the many known acyl-CoA utilizing enzymes, we have chosen acyl-CoA oxidase from *Arthrobacter* as a test enzyme for our photoaffinity probes. This enzyme is commercially available in relatively pure form (Figure 1B). The enzyme is composed of four identical subunits, each having a molecular weight of 54 000. The enzyme efficiently utilizes acyl-CoAs of various chain lengths as well as azidoacyl-CoA analogs. Further studies using our analogs should yield specific information about the active site of acyl-CoA oxidase.

We have synthesized [³²P]-labeled CoA of high specific activity using dephospho-CoA kinase. Two photoreactive acyl-CoA analogs have also been synthesized and demonstrated to be recognized by the active site of acyl-CoA oxidase. These photoaffinity labels will be useful in studying plant lipid metabolism as well as general acyl-CoA binding proteins.

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