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Coenzyme A Analogs. II. Enzymatic Conversion of D-Oxypantetheine 4′-Phosphate to Oxy-Coenzyme A*

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ABSTRACT: The enzymatic conversion of D-oxypantetheine 4′-phosphate to oxy-coenzyme A was investigated with coenzyme A synthesizing enzymes isolated from beef liver. DEAE-cellulose column chromatography of the enzymatic incubation mixtures containing ATP-8-^{14}C and D-oxypantetheine 4′-phosphate separated a radioactive component identical with synthetic oxy-coenzyme A. Dephosphooxy-coenzyme A was enzymatically (a) cleaved by pyrophosphate to oxypantetheine 4′-phosphate and ATP and (b) phosphorylated by ATP to oxy-coenzyme A. Enzymatically prepared oxy-coenzyme A possessed the same inhibitory action as the synthetic material in the phosphotransacetylase reaction.

The oxygen analog of coenzyme A, oxy-coenzyme A, has been recently synthesized and shown to be a competitive inhibitor of CoA[1] in the phosphotransacetylase reaction (Stewart and Miller, 1965; Miller *et al.*, 1966). In order to determine if oxy-CoA can be prepared enzymatically, we investigated the ability of the crude CoA-synthesizing enzyme system, isolated from beef liver by the procedure of Hoagland and Novelli (1954), to convert D-oxypantetheine 4′-phosphate to oxy-CoA.

A net synthesis of oxy-CoA was achieved *via* reactions 1 and 2.

oxypantetheine 4′-phosphate + ATP = dephosphooxy-CoA + PP_i (1)

dephosphooxy-CoA + ATP → ADP + oxy-CoA (2)

(net) oxypantetheine 4′-phosphate + 2 ATP → oxy-CoA + ADP + PP_i

Experimental Procedures

Materials. D-Oxypantetheine 4′-phosphate, dephosphooxy-CoA, and oxy-CoA were prepared by the procedures of Miller *et al.* (1966). D-Pantetheine 4′-phosphate was prepared by the procedure of Moffatt and Khorana (1961). ATP-8-^{14}C (lot no. 6501) was purchased from Schwarz Bioresearch, Inc., and unlabeled ATP was purchased from Sigma Chemical Co. DEAE-cellulose (Selectacel standard type) was obtained from Carl Schleicher and Schuell. Phosphotransacetylase (lot no. 06155109) CoA, and acetyl phosphate were purchased from Boehringer Mannheim Corp. Lyophilized *Clostridium kluyveri* cells, as a source of crude transacetylase, and venom phosphodiesterase (*Crotalus adamantens*) were purchased from Worthington Biochemical Corp.

Methods. The Biuret reaction was used for protein determinations (Layne, 1957). Venom phosphodiesterase digestions of CoA and oxy-CoA were done by the previously described procedures (Moffatt and Khorana, 1961; Miller *et al.*, 1966). Phosphotransacetylase assays were performed by the procedure of Stadtman (1952) when preparing the beef liver enzymes, and as modified by Bergmeyer *et al.* (1963) for the inhibition studies. The ^{14}C content of column fractions was determined by adding 0.1-ml aliquots to 14 ml of the aqueous counting medium of Bray (1960) and

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[1] Abbreviations used: oxy-CoA, oxy-coenzyme A; desulfo-CoA, desulfocoenzyme A; dephospho-CoA, 3′-dephosphocoenzyme A; dephosphooxy-CoA, 3′-dephosphooxy-coenzyme A; AMP and ATP, adenosine mono- and triphosphates. The IUB systematic name for each of the following trivial enzyme names employed in this paper is: phosphotransacetylase, acetyl-CoA; orthophosphate acetyltransferase (EC 2.3.1.8); dephospho-CoA pyrophosphorylase, ATP:pantetheine 4′-phosphate adenylyltransferase (EC 2.7.7.3); dephospho-CoA kinase, ATP:dephospho-CoA 3′-phosphotransferase (EC 2.7.1.24).

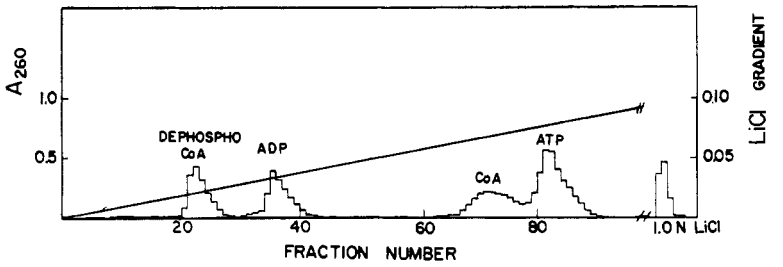

FIGURE 1: DEAE-cellulose column chromatography of pantetheine phosphate; ATP incubation mixture showing net enzymatic synthesis of CoA.

counted in a Packard Tri-Carb liquid scintillation counter. Paper chromatography was carried out by the descending technique on Whatman No. 1 paper, using ethyl alcohol–1 M ammonium acetate, pH 7.5 (7:3) solvent system. ADP and ATP were separated on DEAE-cellulose paper (Whatman Chromedia DE81) by descending elution with 10% aqueous formic acid. Adenine-containing compounds were located by their ability to absorb ultraviolet light (254 mμ). Phosphate-containing compounds were located by the method of Bandurski and Axelrod (1951). Table I lists the R_F values of the various compounds.

TABLE I: R_F Values of Compounds.

Compound	System A[a]	System B[b]
CoA	0.19	
Dephospho-CoA	0.48	
Oxy-CoA	0.16	0.02
Dephosphooxy-CoA	0.45	0.31
Oxypantetheine 4′-phosphate	0.57	—
Adenosine 2′(3′)-diphosphate	0.04	—
Venom digest of Oxy-CoA	0.04, 0.57	—
ATP	0.04	0.02
ADP	0.06	0.24
AMP	0.16	0.89
Peak "X"	0.15	0.34

[a] Ethyl alcohol–1 M ammonium acetate, pH 7.5 (7:3). [b] Formic acid (10%)–DEAE-cellulose paper.

CoA-Synthesizing Enzymes and Incubations. The procedure of Hoagland and Novelli (1954) for the isolation from pig liver of a crude enzyme system containing dephospho-CoA pyrophosphorylase and dephospho-CoA kinase was successfully applied to beef liver. Incubations were carried out in a total volume of 2 ml at 38° for 1.5 hr and contained glycyglycine buffer, adjusted to pH 7.4 with Tris (80 μmoles), cysteine (20 μmoles), $MgSO_4$ (4 μmoles), ATP (5 μmoles), enzyme system (3 mg), and substrate. The substrates were: D-pantetheine 4′-phosphate (4 μmoles), D-oxypantetheine 4′-phosphate (4 μmoles), and dephosphooxy-CoA (2 μmoles). When dephosphooxy-CoA was incubated with 40 μmoles of inorganic pyrophosphate, ATP was omitted. The reactions were stopped by placing the incubation tubes in a boiling water bath for 1 min and removing the precipitated protein by centrifugation.

DEAE-Cellulose Chromatography. The centrifuged incubation mixtures were diluted to a total volume of 25 ml and applied to the top of a 1.3 × 50 cm DEAE-cellulose (chloride form) column. The column was washed with 0.003 N HCl until the effluent was free of ultraviolet-absorbing material. The adsorbed compounds were eluted by application of an acidic lithium chloride linear gradient. The reservoir contained 350 ml of 0.12 N LiCl in 0.003 N HCl and the mixing vessel contained 350 ml of 0.003 N HCl. Fractions (5 ml) were collected at a flow rate of 0.5 ml/min. Each ultraviolet-absorbing peak was pooled. After adjusting the pH to 4.5 with dilute solutions of LiOH, the peaks were evaporated to dryness *in vacuo*. Lithium chloride was removed from the solid white residues by repeated extractions with small volumes of methyl alcohol–acetone (1:15). After drying over P_2O_5 *in vacuo* at room temperature overnight the residual material was dissolved in 1 ml of water and characterized by paper chromatography.

The concentration of each peak was calculated from the optical density units at 260 mμ, assuming an extinction coefficient of 15 × 10^3 for the adenosine moiety.

Results

The validity of our experimental approach was tested by incubating pantetheine 4′-phosphate with ATP. Figure 1 clearly demonstrates the synthesis of CoA, 8.4 ODU, and its effective separation from the products of the enzymatic reaction mixture by DEAE-cellulose column chromatography. In addition to CoA, dephospho-CoA (8.7 ODU) and ADP (8.4 ODU) were produced in the reaction. The 1 N LiCl elution peak contains 5.2 ODU presumably, the mixed disulfide forms of CoA.

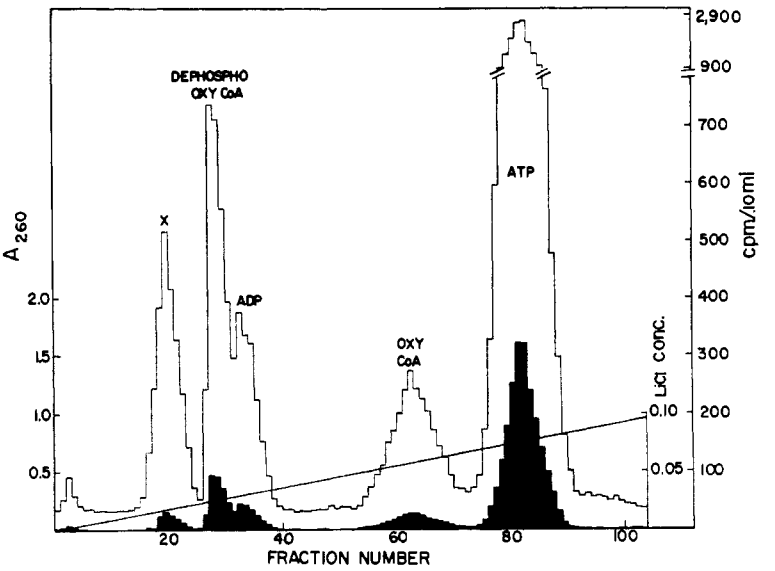

FIGURE 2: DEAE-cellulose column chromatography of oxypantetheine phosphate; ATP incubation mixture showing net enzymatic synthesis of oxy-CoA. The shaded area is the A_{260} elution profile and the upper curve is the elution pattern of radioactivity. Peak X is unidentified.

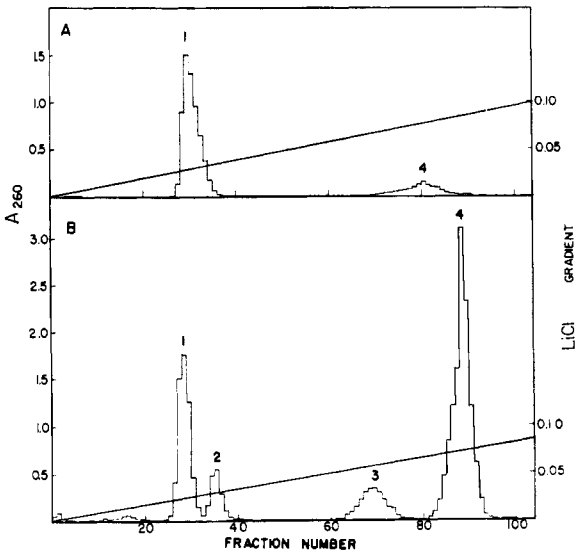

FIGURE 3: DEAE-cellulose column chromatography of dephosphooxy-CoA incubation mixtures. (A) Dephosphooxy-CoA incubated with inorganic pyrophosphate and (B) dephosphooxy-CoA incubated with ATP. Peak 1, dephosphooxy-CoA; peak 2, ADP; peak 3, oxy-CoA; peak 4, ATP.

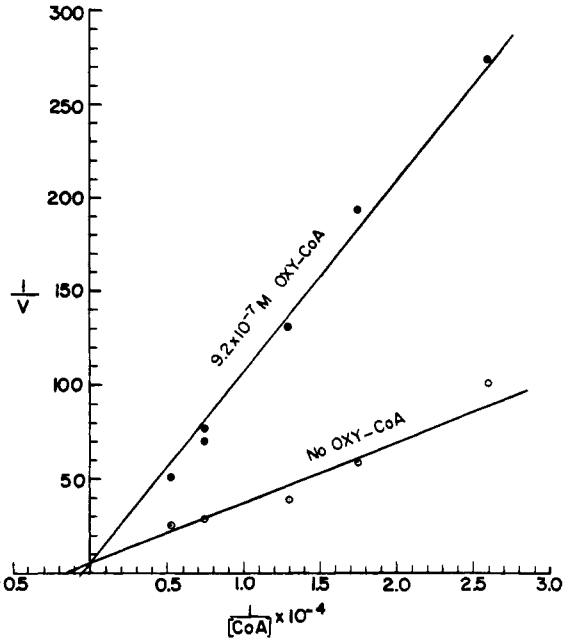

FIGURE 4: Double-reciprocal plot showing competitive inhibition of the phosphotransacetylase reaction by oxy-CoA isolated from peak 3, Figure 3B. The ordinate is the number of seconds required to observe an $OD_{233m\mu}$ change of 0.1, after addition of enzyme. The concentration of oxy-CoA is estimated from its absorbency at 260 mμ.

The elution pattern of the incubation mixture containing oxypantetheine 4′-phosphate and ATP-8-^{14}C is shown in Figure 2. The peaks containing the radioactive label are directly superimposed upon the ultraviolet-absorbing peaks. The yield of radioactive oxy-CoA was 6.5 ODU. The unidentified peak, "X," contained 3.6 ODU. The presence of label, a positive reaction to the phosphate spray reagent, the elution position of "X," and its R_F in system A (*cf.* Table I) implicated AMP. However its R_F on DEAE paper

was not similar to AMP. Paucity of this material hindered further attempts at identification.

The ability of the crude enzyme preparation, containing dephospho-CoA pyrophosphorylase and dephospho-CoA kinase, to cope with the dephosphooxy-CoA is confirmed in Figure 3. The reversibility of the dephospho-CoA pyrophosphorylase reaction is indicated in Figure 3A. Incubation of 2 μmoles of dephosphooxy-CoA with 40 μmoles of sodium pyrophosphate produced 5.8 ODU (0.38 μmole) of ATP. Dephospho-CoA kinase readily catalyzes the phosphorylation of dephosphooxy-CoA by ATP (Figure 3B). The oxy-CoA peak contained 11.2 ODU (0.75 μmole) and was isolated in a 38% yield based upon the substrate concentration of 2 μmoles.

The identity of oxy-CoA isolated from peak 3, Figure 3B, was verified by inhibition studies with the phosphotransacetylase reaction in addition to routine paper chromatography and venom phosphodiesterase digestion. The K_i, 4.2×10^{-7} (Figure 4), is consistent with the value, 3.5×10^{-7}, reported for the crystalline synthetic compound (Miller *et al.*, 1966).

Discussion

This study demonstrates the enzymatic conversion of D-oxypantetheine 4′-phosphate to oxy-CoA. The results indicate that the thiol group is not intimately involved in the *in vitro* conversion of pantetheine 4′-phosphate to CoA by the beef liver system. An ancillary result of this study is the demonstration of a feasible enzymatic route for the synthesis of oxy-CoA or CoA labeled with ^{14}C in the adenosine moiety.

These results also may explain the effect oxypantetheine has on *Lactobacillus helveticus*. Oxypantetheine is a competitive growth inhibitor of this pentetheine-requiring microorganism (Stewart *et al.*, 1955; Pierpoint *et al.*, 1955). It is probable that oxypentetheine inhibits the growth of *L. helveticus* by an *in vivo* synthesis of oxy-CoA. While not proving this proposition, this study supports it, especially if oxy-CoA *in vivo* has as broad an enzyme spectrum of CoA antagonism as Chase *et al.* (1966) have reported for desulfo-CoA *in vitro*.

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