FORMATION OF 3'-^{32}P-ACYL CoA FROM γ-^{32}P-ATP IN GUINEA PIG LIVER MITOCHONDRIAL EXTRACTS

Frank R. Masiarz, Amiya K. Hajra and Bernard W. Agranoff

Neuroscience Laboratory, Mental Health Research Institute
and Department of Biological Chemistry,
University of Michigan, Ann Arbor, Michigan 48104

Received December 13, 1971

Summary: A soluble extract that catalyzes the exchange of acyl groups from acyl coenzyme A to coenzyme A has been obtained from guinea pig liver mitochondria. None of several known acyl group acceptors stimulates the transfer as well as does coenzyme A itself. The preparation also contains dephosphocoenzyme A phosphokinase activity and thus provides a convenient system for the preparation of 3'-^{32}P-acyl coenzyme A from γ-^{32}P-ATP.

During the course of experiments designed to demonstrate the labeling of acidic phospholipids in mitochondria by γ-^{32}P-ATP, we found that a soluble extract of mitochondria, in the presence of a mitochondrial lipid extract catalyzed the formation of labeled material, which by several criteria appeared to be a single lipid. It has been identified as 3'-^{32}P-acyl coenzyme A and we report here some properties of the labeling system.

MATERIALS AND METHODS

A 10% homogenate of fresh guinea pig liver was prepared in chilled 0.25 M sucrose containing 2 mM Na_2 EDTA. All subsequent steps were performed at 0-2° unless otherwise stated. After centrifugation at 800xg for 10 min, the supernatant was recentrifuged at 8700xg for 10 min. The mitochondrial pellet was washed once with 5 ml of 0.25 M sucrose per g of liver and then suspended in 2 ml of water per g of liver and frozen in a dry ice-acetone bath. After thawing in a 30° bath, the suspension was quickly cooled to 2° and centrifuged at 100,000xg for 60 min and the supernatant carefully removed and used as the source of enzyme activity. After dialysis against 10 mM Tris-HCl buffer (pH

7.4) overnight, the preparation was lyophilized in 10 ml portions and stored at -20° with little loss of activity for several weeks. There was a 4-fold variation in initial activity from preparation to preparation. The pellet from the enzyme preparation was heated at 100° for 15 min in a water bath, homogenized in 1 ml of 0.25 M sucrose per g of liver, and termed the heated residue. The lipid extract was prepared by suspending 0.4 ml of the heated residue in 0.8 ml of water, followed by 4.5 ml of chloroform-methanol (1:2), 1.5 ml of chloroform, and 1.5 ml of 2 M KCl. The mixture was stirred well, centrifuged, the lower layer removed, dried under N_2, and sonicated in 0.4 ml of water. Incubation mixtures contained either γ-^{32}P-ATP or 3'-^{32}P-palmityl CoA as labeled precursors. The composition of the reaction mixtures is given in the Tables. Incubations with γ-^{32}P-ATP were stopped by the addition of 13.5 ml of chloroform-methanol (1:2) and lipid was extracted as described previously (1). An aliquot of the lower layer was dried and counted in a toluene scintillant containing 5% ethanol. Incubations containing 3'-^{32}P-palmityl CoA were stopped by freezing in a dry ice-acetone bath. Aliquots of the incubation mixtures were submitted to high-voltage tank electrophoresis using Whatman #1 paper and 0.06 M sodium oxalate buffer (pH 1.5) for 40 min at 4000V. After oven drying, the paper was submitted to radioautography overnight for localization of labeled compounds. The system resolved completely ATP, CoA, P_i, ADP (3'5' or 5') and acyl CoA. Areas of the paper containing compounds of interest were minced into scintillation vials containing 0.5 ml of water and counted in 10 ml of a toluene scintillant containing 16.7% BBS-3 (Beckman). Silica gel H-oxalate TLC plates were developed in chloroform-methanol-water-concentrated aqueous NH_3 (90:90:22:8) (2) and submitted to radioautography, followed when appropriate by spraying with Dittmer phosphate reagent (3). Additional chromatography was performed using the systems of Bressler and Wakil (4) and Ullman and Radin (5). γ-^{32}P-ATP was prepared from carrier-free $H_3{}^{32}PO_4$ by the method of Glynn and Chappel (6). Acyl CoA was isolated from incubation mixtures by the method of Galliard and Stumpf (7), using Celite in place of glass wool. A preparative incubation contained 1.75

ml of 1 M Tris-HCl (pH 7.4), 1.75 ml of 0.1 M $MgCl_2$, 1.75 ml of 0.1 M NaF, 7 ml of enzyme preparation (~28 mg protein), 1.4 mg of dephospho-CoA, 3.5 ml of γ-^{32}P-ATP (1.5×10^9 cpm, 4.3×10^8 cpm/μmole) and water to a volume of 20.3 ml. After incubation at 37° for 20 min, 0.7 ml of palmityl CoA (2 mg/ml) was added and the incubation was continued for 5 min before the addition of an equal volume of cold 1% perchloric acid. Protein was determined by the biuret method (8). Coenzyme A and its derivatives were obtained from P-L Laboratories.

Table I. Formation of Labeled Lipid from γ-^{32}P-ATP

The system contained Tris-HCl buffer (pH 7.4, 0.1 mmole), $MgCl_2$ (0.01 mmole), NaF (0.01 mmole), γ-^{32}P-ATP (~4×10^7 cpm, 0.1 μmole), enzyme preparation (1.6 mg protein in 0.4 ml) and additions in a total volume of 1.2 ml. After 10 min at 37°, incubations were terminated by the addition of chloroform-methanol (1:2).

Incubations	Labeled lipid formed cpm
Expt 1	
Enzyme alone	400
Heated residue (0.4 ml) alone	100
Enzyme + heated residue (0.4 ml)	5,500
Expt 2	
Boiled enzyme + heated residue (0.4 ml)	100
Heated residue (0.4 ml) alone	100
Enzyme + heated residue (0.4 ml)	6,500
Enzyme + lipid extract (0.4 ml)	8,500
Expt 3	
Enzyme alone	800
Enzyme + lipid extract (0.4 ml)	4,200
Enzyme + palmityl CoA (0.1 μmole)	14,250

RESULTS

Table I summarizes typical experiments from incubations using γ-^{32}P-ATP, and indicates the marked stimulatory effect of the heated residue and lipid extract. Palmityl CoA fully replaced the mitochondrial lipid extract in providing maximal stimulation of the reaction.

The product of the reaction was identified by several methods and found to have the same properties in each of the experiments of Table I. On electrophoresis, the product adhered completely to the origin when protein was present, as did authentic palmityl CoA; in the absence of protein, both streaked up from the origin. It co-migrated with authentic palmityl CoA in the three chromatography systems used. The product was eluted from a Unisil

Table II. Formation of Labeled Palmityl CoA from γ-^{32}P-ATP

The incubation mixture was as described in Table I.

	Additions	cpm
Expt 1		
	None	1,720
	+ Palmityl CoA (0.1 µmole)	31,060
	+ Palmityl CoA (0.1 µmole) + CoA (0.1 µmole)	7,400
	+ Palmityl CoA (0.1 µmole) + dephospho-CoA (0.1 µmole)	42,360
Expt 2		
	None	50
	+ Palmityl CoA (0.1 µmole)	6,200
	+ CoA (0.1 µmole)	100
	+ CoA (0.1 µmole) + palmitic acid (0.1 µmole)	50
	+ CoA (0.1 µmole) + palmityl CoA (0.1 µmole)	1,050
	+ Dephospho-CoA (0.1 µmole)	100
	+ Dephospho-CoA (0.1 µmole) + palmitic acid (0.1 µmole)	125
	+ Dephospho-CoA (0.1 µmole) + palmityl CoA (0.1 µmole)	10,350

column with chloroform-methanol (1:1) and, after alkaline methanolysis, radioactivity was quantitatively released as $^{32}P_i$ by treatment with alkaline phosphatase. These data, in addition to data from further chemical degradation studies, led to the identification of the product as 3'-^{32}P-acyl CoA.

Electrophoresis of aliquots from γ-^{32}P-ATP incubation mixtures revealed that in addition to acyl CoA, CoA was labeled as well. The most reasonable explanation for the labeling of CoA appeared to be a result of the presence of dephospho-CoA and its kinase in our preparation. The eventual acylation of the 3'-^{32}P-CoA would then yield labeled acyl CoA. Table II summarizes the effects of the addition of several substances on the labeling of acyl CoA. The enzyme preparation in experiment 1 was unusually active.

Consistent with the hypothesis that dephospho-CoA was converted to labeled CoA was the finding that the labeling of acyl CoA was stimulated by the addition of dephospho-CoA and inhibited by the addition of CoA. The addition of CoA and fatty acid had no stimulatory effect. Addition of dephospho-CoA and fatty acid also had no effect on the labeling of acyl CoA. In a separate experiment, the addition of dephospho-CoA stimulated the labeling of CoA by 270%.

This system provided us with a simple method for the preparation of 3'-^{32}P-acyl CoA, which was then isolated as stated in MATERIALS AND METHODS and used in further experiments. A typical preparation of 3'-^{32}P-acyl CoA was

Table III. Formation of Labeled CoA from 3'-^{32}P-Palmityl CoA

The system contained Tris-HCl buffer (pH 7.4, 0.025 mmole), $MgCl_2$ (0.0025 mmole), NaF (0.0025 mmole), 3'-^{32}P-palmityl CoA (0.04 µmole, 100,000 cpm in Expt 1, 50,000 cpm in Expt 2, 3 and 4), and 0.4 mg of protein from 10^5xg supernatant in 0.1 ml in a total volume of 0.35 ml. In all of the experiments a substrate blank is included which contains 0.1 ml of bovine serum albumin (10 mg/ml) instead of the enzyme preparation. Tubes were incubated at 37° for 5 min, frozen immediately and aliquots submitted to electrophoresis and radioautography.

	Additions	cpm
Expt 1		
	Substrate blank	1,500
	None	4,275
	+ CoA (0.013 µmole)	34,500
	+ Dephospho-CoA (0.015 µmole)	7,320
Expt 2		
	Substrate blank	1,100
	None	7,150
	+ CoA (0.026 µmole)	15,950
	+ 3'5' ADP (0.5 µmole)	8,470
	+ 3'5' ADP (0.5 µmole) + CoA (0.026 µmole)	18,700
Expt 3		
	Substrate blank	990
	None	2,530
	+ CoA (0.032 µmole)	4,620
	+ acetyl CoA (0.3 µmole)	2,860
	+ DL-carnitine (0.025 µmole)	2,750
	+ DL-carnitine (0.25 µmole)	3,630
	+ DHAP (0.125 µmole)	2,200
	+ Lyso PhA (0.025 µmole)	2,860
Expt 4		
	Substrate blank	900
	None	2,650
	+ CoA (0.032 µmole)	6,020
	+ DL-carnitine (0.150 µmole)	3,710
	+ CoA (0.032 µmole) + DL-carnitine (0.150 µmole)	6,980

96% pure radiochemically, and the yield from 1.4×10^9 cpm of γ-^{32}P-ATP was 2.6×10^7 cpm (1.9%). With this substrate, investigation of the reverse reaction (the production of CoA from acyl CoA) was carried out. Table III summarizes a number of experiments designed to examine several possible reaction mechanisms. Formation of labeled CoA was strongly stimulated by the addition of CoA but dephospho-CoA's effect was clearly weaker. The addition of 3'5' ADP, the labeled nucleotide portion of acyl CoA, did not affect the labeling of CoA. An inhibitory effect would have been expected if the reaction involved exchange *via* cleavage of the pyrophosphate bond of CoA. These experiments led us to the conclusion that 3'-^{32}P-CoA was the acceptor of acyl groups from pre-existing acyl CoA in the preparation.

Experiments in which the specificity of CoA as an acyl group acceptor was examined indicate that of the number of substances tested, dephospho-CoA or high levels of DL-carnitine are weakly effective, while DHAP and lyso-PhA are inactive.

DISCUSSION

The data reported indicate that the mitochondrial supernatant fraction of guinea pig liver is capable of catalyzing two reactions of interest. The first is the phosphorylation of dephospho-CoA by ATP:

$$(1) \quad \text{dephospho-CoA} + \text{ATP}^{*} \longrightarrow \text{CoA}^{*} + \text{ADP}$$

This reaction has been studied and an enzyme catalyzing it has been purified from rat liver (9). The second reaction is somewhat unusual and, to our knowledge, has not been described previously:

$$(2) \quad \text{acyl CoA} + \text{CoA}^{*} \rightleftharpoons \text{acyl CoA}^{*} + \text{CoA}$$

This reaction appears to be freely reversible. Our observation reflects the ability of the preparation to produce labeled CoA from γ-^{32}P-ATP, which then freely exchanges with unlabeled acyl CoA, when added either as a component of the mitochondrial lipid extract or in chemically pure form.

We are led to conclude that the stimulation of acyl CoA labeling from labeled CoA by the addition of acyl CoA reflects acyl group transfer. The production of labeled acyl CoA from labeled CoA is not likely due to thiokinase activity, since the addition of fatty acid and dephospho-CoA was not stimulatory in γ-^{32}P-ATP incubations. The involvement of carnitine acyl transferase is not likely, since the addition of carnitine had no appreciable effect on the release of CoA from acyl CoA. CoA and carnitine appear to have additive effects on the release of CoA and, therefore, are probably not influencing the same reaction. No other acyl group acceptor tested, including dephospho-CoA, functioned as well as CoA in stimulating the exchange. Studies with ^{35}S-acyl CoA support our conclusion that acyl group exchange occurs under our experimental conditions. Other preliminary studies indicate that acetoacetyl CoA is also labeled in γ-^{32}P-ATP incubation mixtures, but the involvement of succinyl CoA:3-keto acid coenzyme A transferase is not likely, since the

established reaction mechanism precludes an exchange of CoA (10).

The physiological significance of this novel enzymatic exchange reaction is unknown. It may reflect the presence of activity for an as yet unidentified acyl group acceptor. Alternatively, the enzyme may be a component of a transmembrane acyl displacement mechanism analogous to that seen for carnitine (11).

Acknowledgements. We thank Mr. Edward Seguin for capable technical assistance. This work was supported by Grant NB 3101 of the National Institutes of Health. F. R. M. is supported by USPHS Training Grant GM 00187.

REFERENCES

1. Hajra, A. K., Seguin, E. B. and Agranoff, B. W., J. Biol. Chem., 243, 1609 (1968).
2. Gonzalez-Sastre, F. and Folch-Pi, J., J. Lipid Res., 9, 532 (1968).
3. Dittmer, J. C. and Lester, R. L., J. Lipid Res., 5, 126 (1964).
4. Bressler, R. and Wakil, S., J. Biol. Chem., 237, 441 (1962).
5. Ullman, M. D. and Radin, N. S., J. Lipid Res. (1972) in press.
6. Glynn, I. M. and Chappell, J. B., Biochem. J., 90, 147 (1964).
7. Galliard, T. and Stumpf, P. K., Biochem. Prep., 12, 66 (1968).
8. Gornall, A. G., Bardawill, C. S. and David, M. M., J. Biol. Chem., 177, 751 (1949).
9. Suzuki, T., Abiko, Y. and Shimizu, M., J. Biochem., 62, 642 (1967).
10. Hersh, L. B. and Jencks, W. P., J. Biol. Chem., 242, 3468 (1967).
11. Fritz, I. B., Advan. Lipid Res., 1, 285 (1963).