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EVIDENCE AGAINST PHOSPHORYLATION OF ADP BY OLEOYL PHOSPHATE CATALYZED BY SUBMITOCHONDRIAL PARTICLES

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SUMMARY

When oleoyl phosphate and ADP were incubated with heart submitochondrial particles in the presence of glucose-hexokinase trap according to a reported procedure [Griffiths, D.E. (1976) Biochem. J. 160: 809-812], a 10% yield of glucose-6-phosphate was detected by chemical analysis. Although lower concentration of oleoyl phosphate improved the yield to 80-85%, the mode of formation of glucose-6-phosphate was not clear under the experimental condition used to improve the yield. In order to test decisively whether the phosphoryl group of oleoyl phosphate was transferred to ADP to form ATP which was estimated in the form of glucose-6-phosphate, [^{32}P]oleoyl phosphate was synthesized. The use of isotopically labelled oleoyl phosphate showed only about 5% yield of [^{32}P]glucose-6-phosphate by paper chromatographic analysis, whereas chemical analysis of the same system gave 80% yield of glucose-6-phosphate. Such an observation demonstrated that glucose-6-phosphate estimated by chemical assay is not the result of phosphorylation of ADP with oleoyl phosphate catalyzed by the submitochondrial particles.

INTRODUCTION

Oleoyl phosphate-dependent ATP synthesis has been reported by Griffiths and co-workers (1,2) to be a representative of the terminal reaction of oxidative phosphorylation in all bioenergetic membranes. Johnston and Criddle (3) arrived at a similar conclusion with F_1-ATPase from yeast mitochondria. In this work, it was found that bovine heart submitochondrial particles in the presence of glucose-hexokinase trap (1,2) gave only a 10% yield of glucose-6-phosphate (G6P) as estimated from the increase in fluorescence accompanying the conversion of $NADP^+$ to NADPH in the presence of G6P dehydrogenase (4). The use of lower concentration of oleoyl phosphate (0.1 mM——0.5 mM) improved the yield. However, the amount of G6P formed was less than the amount of ADP used in the

Abbreviations: DMF, N,N-dimethylformamide; G6P, glucose-6-phosphate; ETPH, electrotransport particles (heavy, phosphorylating)

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reaction system. Consequently, the possibility of stimulated disproportionation of ADP could not be ruled out.

In order to see if oleoyl phosphate participated directly with ADP to synthesize ATP in the presence of the submitochondrial particles, [^{32}P]oleoyl phosphate was synthesized. Using [^{32}P]oleoyl phosphate, the resulting yields of [^{32}P]G6P by paper chromatographic analysis (5) were compared with those of G6P by chemical assay in the same system.

MATERIALS AND METHODS

Oleoyl chloride, acetyl phosphate, substrates and coenzymes were obtained from Sigma Chemical Company. Efrapeptin was a generous gift from Dr. R.L. Hamill of Lilly Research Laboratories. Anhydrous phosphoric acid was purchased from ICN Pharmaceutical Inc. Tetrabutyl ammonium hydroxide was obtained from Eastman Organic Chemicals. $^{32}P_i$ and [8-^{14}C]ADP were from New England Nuclear.

Submitochondrial particles (Mn^{++}, Mg^{++}) from bovine heart mitochondria were prepared according to the procedure described by Beyer (6). G6P and ADP were estimated fluorometrically (4). [^{32}P]G6P, [^{14}C]ADP and [^{14}C]AMP were analyzed by paper chromatography (5). Radioactivity was measured directly from the paper in 1-cm strips in the presence of 5.0 ml Aquasol by means of a Liquid Scintillation System with 90% counting efficiency for ^{32}P and 60% for ^{14}C. Reversed Electron Transport and ATPase activities were measured at 30°C according to described procedures (7,8). Complex V was isolated according to the procedure of Hatefi, et al. (9).

Oleoyl phosphate was prepared according to reported procedures (2,3) and also synthesized from tetrabutyl ammonium phosphate (1 mmol) and oleoyl chloride (1 mmol) in anhydrous DMF (25 ml) by stirring at 4° for 10-12 hrs. Evaporation of DMF under reduced pressure gave a thick liquid residue which was extracted with anhydrous ether. Evaporation of ether under rotary evaporator gave the required product. The infrared spectrum of the liquid was free from absorbance at 1800 cm^{-1}, characteristic of acyl chloride. A sample in anhydrous DMF was quantitated by hydroxylamine assay (10). The yield of the reaction was 90-95%. Thin layer chromatography of a sample in silica gel gave R_F 0.4 (3). [^{32}P]-oleoyl phosphate was prepared from [^{32}P]phosphoric acid by the same procedure.

Tetrabutyl ammonium phosphate was prepared by reacting the corresponding hydroxide in methanol (25% solution) with anhydrous phosphoric acid in 1:1 molar ratio. Methanol was evaporated under reduced pressure. The salt was dried in vacuum desiccator over phosphorous pentoxide for 24 hrs before reacting with oleoyl chloride.

RESULTS AND DISCUSSION

The important observations of oleoyl phosphate-dependent ATP synthesis by various energy-transducing membranes and F_1-ATPase from yeast mitochondria (1,2,3) prompted us to examine similar behavior in F_1 and complex V isolated from beef heart mitochondria. Following reported procedures, attempts to

detect ATP synthesis both by glucose-hexokinase trap and with [^{14}C]ADP by ascending chromatography on polyethyleneimine paper met with failure.

We next examined ETPH (Mn^{++}, Mg^{++}) in the presence of glucose-hexokinase trap. Reversed Electron Transport and ATPase activities of ETPH preparation were 200 nmol min^{-1} mg^{-1} protein and 0.8 µmol min^{-1} mg^{-1} protein respectively at 30°C. Chemical assay detected 10% yield of G6P from 1 mM oleoyl phosphate. The blank of the method was 23 nmol mg^{-1} protein. The yield of G6P formation improved on decreasing oleoyl phosphate and increasing ADP concentrations (Table 1). But the blank of the method also increased simultaneously from 13 nmol mg^{-1} protein for 0.1 mM ADP to 47 nmol mg^{-1} protein for 0.6 mM ADP.

Because of the precipitation caused by 1mM oleoyl phosphate, we had to use a lower concentration (0.1 mM). In order to have appreciable counts in the final samples, the concentration of [^{32}P]oleoyl phosphate was varied from 0.2 mM to 0.5 mM. To check if oleic acid accompanying oleoyl phosphate was responsible for precipitation at 1 mM concentration, oleoyl phosphate was synthesized by three independent methods. The procedures of Griffiths and co-workers (2) as well as our own gave 90-95% yield whereas that prepared by the method of Johnston and Criddle (3) gave only 60% yield. Unreacted oleoyl chloride was indicated by the infrared spectrum (1800 cm^{-1}) along with the product by the latter method. However, using different methods did not change the overall result.

Reduction of ADP concentration from 0.2 mM to 0.02 mM cut down the yield of G6P formation drastically (Table 1). Endogenous ADP concentration in the ETPH preparation was estimated to be 29 nmol mg^{-1} protein. In the absence of exogenous ADP only 5 nmol G6P could be detected per mg protein. Dicyclohexyl-carbodiimide lowered the yield partially from 79% to 20% but did not completely inhibit as reported (2).

In order to test decisively if G6P formed was the result of direct participation of oleoyl phosphate with ADP in the presence of ETPH, we synthesized [^{32}P]oleoyl phosphate. The use of isotopically labelled oleoyl

TABLE 1

Quantitation of G6P by Fluorometric Analyses (4)

oleoyl phosphate (μ mole)	ADP (μmole)	G6P (nmol/20 min/mg protein)	% yield[a] (nmol G6P x 100/nmol oleoyl phosphate)
0	0.2	23	——
1.0	0.2	123	10
0.5	0.2	133	22
0.1	0.2	63	40
0.5	0.1	73	12
0.5	0.2	133	22
0.5	0.6	363	63
0	0.2	27	——
0.1	0.2	106	79
0.1 + DCCD (1 μ g/mg)	0.2	47	20
0	0	4.4	——
0.1	0	7.1	2.7
0	0.02	5.5	——
0.1	0.02	12.1	6.6

[a]% yield was calculated after subtracting the blank obtained by adding an equivalent amount of DMF (25 μl/ml) without oleoyl phosphate.

ETPH (1.0 mg protein) inhibited with rotenone (1 μg/mg protein) and antimycin A (1 μ g/mg protein) was added in sucrose (250 mM), Tris chloride (10 mM, pH 7.7) EDTA (1 mM) buffer (50 μ l) to 1 ml of the phosphorylating medium containing 250 mM sucrose, 22 mM glucose, 5 mM $MgCl_2$, 0.5 mM EDTA, 20 mM Tris chloride, pH 7.3 and the stated amounts of oleoyl phosphate and ADP.

In the lower half of the table, the buffering capacity of the phosphorylating medium was increased from 20 mM Tris hydrochloride to 40 mM, pH 7.5.

phosphate under similar reaction conditions gave only a 5% yield of [^{32}P]G6P according to paper chromatographic analysis. Estimation of G6P in the same system by chemical analysis gave much higher yield (Table 2). These and other observations of similar results indicate that the high yield of G6P estimated by chemical analyses is not the result of direct participation of oleoyl phosphate with ADP in the reacting system.

Table 3 contains some of the results obtained by paper chromatographic analysis. The concentration of ADP was raised five-fold (0.2 mM to 1.0 mM) in order to see if more [^{32}P]G6P could be obtained. No chemical assay was

TABLE 2

Quantitation of Glucose-6-phosphate (nmol/20 min/mg protein) by Chemical Assay and Paper Chromatographic Analysis Using [^{32}P]Oleoyl Phosphate

Reaction System	Chemical Assay		Paper Chromatography	
	nmole/mg	% yield	nmole/mg	% yield
Complete	194	88	9.0	4.1
Complete minus ADP	4.3	1.9	8.5	3.9
Complete minus ETPH	21.5	9.7	9.8	4.4

The reaction system is the same as in the lower part of Table 1. The concentrations of [^{32}P]oleoyl phosphate and ADP were 0.22 mM and 0.2 mM respectively.

performed on systems containing 1.0 mM ADP, since the blank was too high. The data in Table 2 and Table 3 show that the yield of [^{32}P]G6P was not improved by the increased concentration of ADP.

Different concentrations of P_i were added to the complete system to test whether the small percentage of [^{32}P]G6P, as assayed by paper chromatography, was due to the direct phosphorylation of ADP by [^{32}P]oleoyl phosphate or due to $^{32}P_i$ from the hydrolysis of [^{32}P]oleoyl phosphate. If phosphorylation went through $^{32}P_i$ from [^{32}P]oleoyl phosphate hydrolysis, the addition of 50 mM carrier P_i to the complete system would have lowered the yield of [^{32}P]G6P significantly by isotopic dilution. The experimental results indicate that phosphorylation did not go through the exchange reaction with $^{32}P_i$ from [^{32}P]oleoyl phosphate. Direct phosphorylation by [^{32}P]oleoyl phosphate was also tested by withdrawing [^{32}P]oleoyl phosphate from the system and replacing it by 0.25 mM $^{32}P_i$ (concentration equivalent to 50% hydrolysis of 0.5 mM [^{32}P]oleoyl phosphate). Under such a condition the system failed to give appreciable amount of [^{32}P]G6P.

TABLE 3

Effect of Protein and Inorganic Phosphate on the Yield of [^{32}P]G6P Assayed by Paper Chromatography

Reaction System	[^{32}P]G6P (nmol/20 min/mg protein)	% yield
Complete	27	4.9
Complete minus Hexokinase and ETPH	65	11.8
Complete minus Hexokinase	19	3.8
Complete minus ETPH	27	4.9
Complete + P_i(5 mM, pH 7.5)	50	9.1
Complete + P_i(50 mM, pH 7.5)	46	8.4
Complete + P_i(50 mM, pH 7.5) + Efrapeptin (5 μg/mg)	32	4.8
Complete minus [^{32}P]oleoyl phosphate + P_i(0.25 mM, pH 7.5) + $^{32}P_i$	0.5	0.09

The complete system is identical to that in the lower part of Table 1. The concentrations of [^{32}P]oleoyl phosphate and ADP were 0.55 mM and 1.0 mM respectively.

Only 50% of total radioactivity was found in the supernatant after centrifugation of the perchloric acid treated reaction system. The pellet was highly radioactive. It was not clear whether the radioactivity in the pellet was from radioactively labelled protein resulting from reaction with [^{32}P]-oleoyl phosphate or from the precipitation of [^{32}P]oleoyl phosphate buried inside the pellet.

The results in Table 3 indicate direct phosphorylation of glucose by [^{32}P]oleoyl phosphate. The yield of such phosphorylation was lowered by the presence of hexokinase and/or ETPH. Moreover, as in Table 2, there was no difference in the amount of [^{32}P]G6P formed between the complete and the system with the G6P trap but without ETPH.

Table 4 shows quantitative conversion of ADP to AMP as assayed by paper chromatographic analysis. The conversion appears to be stimulated by oleoyl phosphate.

TABLE 4

Quantitation of AMP (nmole/20 min/mg protein) by Paper Chromatographic Analysis Using ^{14}C-ADP and Oleoyl Phosphate

Reaction System	% ADP at the end of 20 min.	% conversion to AMP	nmole AMP/mg
Complete	1.5	99	248
Complete minus ADP	9.4	76	3.0
Complete minus ETPH	91	7.5	18.8
Complete minus oleoyl phosphate	3.7	87	209

The complete system is the same as in Table 2 except [8-^{14}C]ADP (0.25 mM) and non-radioactive oleoyl phosphate (0.2 mM) were used. [8-^{14}C]ADP (50μCi/μmol) was found to be 99% pure by paper chromatographic analysis.

These data show that the G6P obtained in the above experiments as determined by chemical assay was not the result of direct phosphorylation of ADP by oleoyl phosphate catalyzed by submitochondrial particles.

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