ROLE OF ATP IN THE ATP CITRATE LYASE REACTION

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ATP citrate lyase, which is known as the citrate-cleavage enzyme, catalyzes the following irreversible reaction:

$$\text{Citrate} + \text{ATP} + \text{CoA} \xrightarrow{Mg^{++}} \text{acetyl-CoA} + \text{oxaloacetate} + \text{ADP} + \text{Pi}$$

This enzyme has been found to occur in the soluble fraction from a variety of animal tissues (Srere, 1959). Recently, knowledge on the physiological significance of ATP citrate lyase has increased, especially with regard to its role in lipogenesis (Spencer et al., 1964; Takeda et al., 1964) and in gluconeogenesis (D'Adamo, Jr. and Haft, 1965). With partially purified preparations of enzyme from chicken liver, Eggerer and Remberger (1963), and Srere and Bhaduri (1964) studied the mechanism of this enzyme reaction and suggested that a single protein or tightly bound protein complex catalyzes the overall reaction and that free or enzyme-bound citryl-CoA might be an intermediate in the reaction. However, their suggestion seems to lack convincing evidence and the detailed mechanism of this enzyme reaction is still obscure. In earlier studies in this laboratory, ATP citrate lyase was purified from rat liver as a single homogeneous protein and some of its properties have been presented (Inoue et al., 1966). With this purified preparation, the mode of action of this enzyme, especially with regard to the role of ATP, was investigated and the results of these experiments are

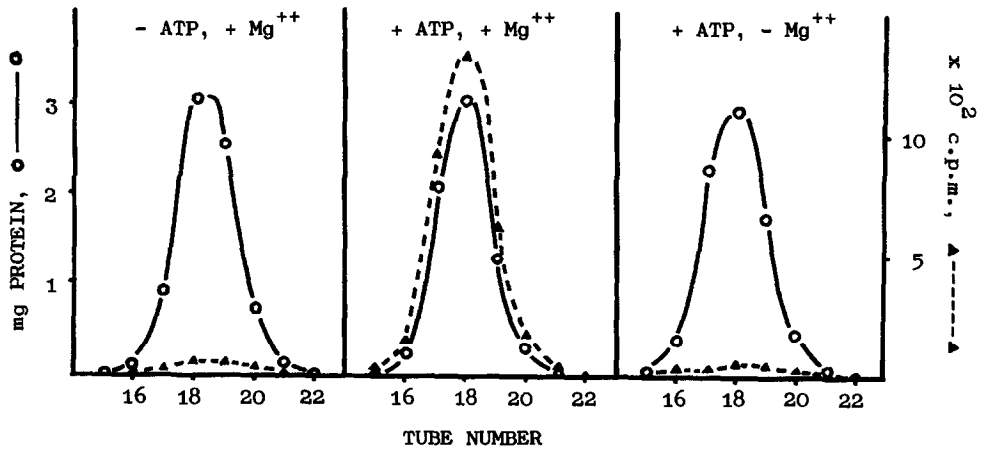

Fig. 1. Formation of enzyme-citrate complex. The reaction mixture contained (in μmoles): 1,5-C^{14}-citrate 2 (2.4×10^6 c.p.m.), ATP 5, $MgCl_2$ 10, 2-mercaptoethanol 10, Tris-HCl buffer (pH 8.0) 100 and enzyme (9 mg protein) in a total volume of 1 ml. ATP or Mg^{++} was omitted as indicated. After incubation at 37° for 10 min., the reaction mixture was chilled and then passed through a column of Sephadex G-50 (1.5 x 30 cm), which had been equilibrated with 0.05 M Tris-HCl buffer (pH 8.0) containing 0.01 M 2-mercaptoethanol. The column was eluted with the same buffer and 1-ml fractions were collected.

presented in this report.

The preparation of ATP citrate lyase used in the present experiments was purified from rat liver as described previously (Inoue et al., 1966). Protein concentrations were determined by the method of Lowry et al. (1951) and radioactivities were measured in a Packard Tri-Carb liquid scintillation spectrometer.

The first experiment was on whether the enzyme-citrate complex is formed as an intermediate during the reaction. The enzyme was incubated with 1,5-C^{14}-citrate and after incubation the protein portion was isolated by column chromatography on Sephadex G-50. As shown in Fig. 1, the enzyme-citrate complex was formed only in the presence of both ATP and Mg^{++}. The next experiment was on whether this enzyme-citrate complex is a true intermediate of the reaction. The enzyme-citrate complex, labelled with 1,5-C^{14}-citrate, was isolated on a Sephadex G-50 column and then incubated with and without

Table I. Formation of Acetyl-CoA from the Enzyme-Citrate Complex

Enzyme-citrate complex added	CoA	Acetyl-CoA formed	
		Observed	Calculated
c.p.m.		c.p.m.	c.p.m.
2,000	-	22	-
2,000	+	624	1,000

The reaction mixture contained (in μmoles): CoA 0.8, 2-mercaptoethanol 20, Tris-HCl buffer (pH 8.0) 40 and enzyme-1,5-C^{14}-citrate complex (8.2 mg protein, 2,000 c.p.m.) in a total volume of 2.5 ml. After incubation for 20 min. at 37°, 1 μmole of unlabelled acetate was added. The enzyme was denatured by heating for 5 min. at 100° and the precipitate formed was removed by centrifugation. The acetyl-CoA in the supernatant was hydrolyzed by treatment with 0.01 N KOH for 15 min. at 80° and the acetate thus formed was trapped in KOH by the diffusion method (Lundquist et al., 1961). The purity of the acetate was checked by thin layer chromatography on silica gel paper (Prey et al., 1962).

CoA. The acetyl-CoA formed was converted to acetate chemically and the acetate was isolated. As shown in Table I, acetyl-CoA was formed from the enzyme-citrate complex only in the presence of CoA. Neither ATP nor Mg^{++} is necessary in this conversion. Therefore, it is evident that the ATP citrate lyase reaction involves the formation of the enzyme-citrate complex as a reaction intermediate.

The next experiment was to study the role of ATP in the formation of the enzyme-citrate complex and the interaction between ATP and the enzyme in the absence of citrate. Thus, ATP-8-C^{14} and ATP-γ-P^{32} were separately incubated with the enzyme in the presence of Mg^{++}, and then the protein was isolated by gel filtration on a Sephadex G-50 column. As shown in Fig. 2, radioactivity associated with the protein was obtained only with ATP-γ-P^{32} and none was found with ATP-8-C^{14}. This indicates that the enzyme-P complex is formed as an activated form of the enzyme. To confirm this, an

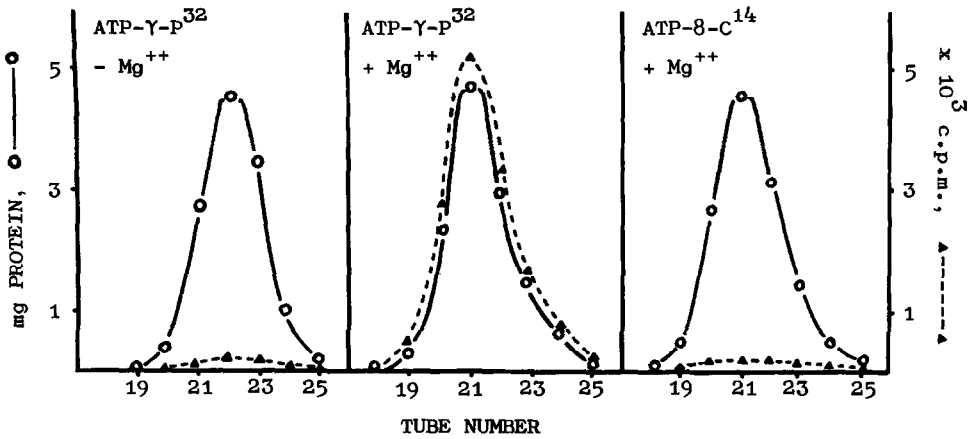

Fig. 2. Formation of enzyme-P complex. The reaction mixture contained (in μmoles): ATP 0.5 (ATP-8-C^{14}, 250,000 c.p.m. or ATP-γ-P^{32}, 226,000 c.p.m.), $MgCl_2$ 5, 2-mercaptoethanol 5, Tris-HCl buffer (pH 8.0) 50 and enzyme (15 mg protein) in a total volume of 0.5 ml. Mg^{++} was omitted as indicated. Incubations were carried out for 5 min. at 37°. Other conditions were as described in Fig. 1.

Table II. ATP-ADP Exchange Reaction

Reaction	ATP formed		
	- Enzyme + Mg^{++}	+ Enzyme + Mg^{++}	+ Enzyme - Mg^{++}
	c.p.m.	c.p.m.	c.p.m.
ATP-Pi exchange*	90	156	
ATP-ADP exchange**	210	27,810	307

The reaction mixture contained (in μmoles): ATP 2, ADP 0.05, Pi 0.05, 2-mercaptoethanol 2, $MgCl_2$ 2, Tris-HCl buffer (pH 8.0) 20 and enzyme (0.75 mg protein) in a total volume of 0.2 ml. Incubations were carried out for 2 min. at 37°. After incubation, the reaction was stopped by addition of perchloric acid to a concentration of 0.2 N. Adenine nucleotides were separated by thin layer chromatography on DEAE cellulose (Randerath, 1962). The areas corresponding to ADP and ATP, respectively, were collected and counted.

* Pi^{32} was used (65,000 c.p.m.).

** ADP-8-C^{14} was used (29,000 c.p.m.).

ATP-ADP exchange experiment was carried out. As shown in Table II, a rapid exchange of isotope between ADP-8-C^{14} and unlabelled ATP was catalyzed by the enzyme, whereas the enzyme was incapable of catalyzing an exchange between Pi and ATP. Therefore, it is clear that the terminal phosphate of ATP is transferred to the enzyme during the reaction, with the formation of a high energy linkage, and that this activation process is reversible. Both enzyme-P formation and the ATP-ADP exchange reaction depend on the presence of Mg^{++} (Fig. 2 and Table II).

The next question was whether this enzyme-P complex is the true intermediate of the reaction. Accordingly, the enzyme-P complex, labelled with P^{32}, was isolated on a Sephadex G-50 column and then incubated with 1,5-C^{14}-citrate in the presence of CoA, and the formation of oxaloacetate was determined. As shown in Table III, oxaloacetate was formed quantitatively as judged by the phosphate bound to the enzyme in the absence of added ATP.

Table III. Formation of Oxaloacetate from Enzyme-P

Expt. No.	P content of enzyme-P used (A)	Oxaloacetate formed from enzyme-P and citrate (B)	B/A
	mμmoles	mμmoles	
1	40.0	38.7	0.97
2	68.5	69.2	1.01

The reaction mixture contained (in μmoles): CoA 0.8, 1,5-C^{14}-citrate 2 (350,000 c.p.m.), sodium oxaloacetate 2, Tris-HCl buffer (pH 8.0) 80, 2-mercaptoethanol 20 and enzyme-P^{32} complex (16 mg protein in Experiment 1 and 16.4 mg protein in Experiment 2) in a total volume of 4.5 ml. After incubation for 15 min. at 37°, the reaction was stopped by addition of 2 N HCl. The oxaloacetate formed was degraded and the $C^{14}O_2$ evolved was trapped and counted as reported previously (Inoue et al., 1966).

Table IV. Release of Pi from Enzyme-P by Citrate

Addition	Pi^{32} released from enzyme-P complex	
	c.p.m.	%
None	116	3.3
Citrate	3,410	98

The reaction mixture contained (in μmoles): potassium citrate 10, 2-mercaptoethanol 5, Tris-HCl buffer (pH 8.0) 20 and enzyme-P^{32} (3,500 c.p.m.) in a total volume of 0.5 ml. After incubation for 20 min. at 37°, perchloric acid was added at 0° to a final concentration of 0.2 N and the protein precipitated was removed by centrifugation. Pi^{32} in the supernatant solution was precipitated as the triethylamine-phosphomolybdate complex (Sugino and Miyoshi, 1964) and the complex was dissolved in 1 N NH_4OH for counting.

Mg^{++} was unnecessary in this conversion. In addition, bound phosphate was almost completely released from the enzyme on addition of citrate, as shown in Table IV.

From these findings, the reaction mechanism of ATP citrate lyase is summarized as follows:

1. Enzyme + ATP $\overset{Mg^{++}}{\rightleftharpoons}$ enzyme-P + ADP
2. Enzyme-P + citrate $\longrightarrow$ enzyme-citrate + Pi
3. Enzyme-citrate + CoA $\longrightarrow$ acetyl-CoA + oxaloacetate + enzyme

Reaction (2) seems to be irreversible or far stronger in the forward direction, because citrate does not stimulate the ATP-Pi exchange reaction.

Details of the present studies will be published elsewhere.

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