SUCCINIC THIOKINASE: EVIDENCE OF A BOUND ^{32}P-3′-DEPHOSPHOCOENZYME A*

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Recent investigations concerning the mechanism of the reaction catalyzed by succinic thiokinase have revealed several new facets of this catalytic process. Upper (1964), after developing an elegant and reproducible purification procedure which provides an essentially homogeneous enzyme from extracts of Escherichia coli, showed formation of a phosphoenzyme in which the phosphoryl group is covalently linked to the protein. Furthermore, he demonstrated that coenzyme A could discharge the phosphoryl group from the enzyme and, based on an exchange of ^{14}C-succinate into succinylCoA, it was suggested that catalysis of the overall process occurs by the following steps:

$$\text{ATP} + \text{enzyme} \rightleftarrows \text{ADP} + \text{phosphoenzyme} \quad (1)$$

$$\text{Phosphoenzyme} + \text{CoA} \rightleftarrows P_i + \text{CoA-enzyme} \quad (2)$$

$$\text{CoA-enzyme} + \text{succinate} \rightleftarrows \text{enzyme} + \text{succinylCoA} \quad (3)$$

Following their observations on an alkali-stable acid-labile bound phosphoryl group in liver mitochondria, Mitchell et al. (1964) found the major portion of their mitochondrial phosphoprotein to be identical with phosphorylated succinic thiokinase and were able to identify the imidazole of histidine as the phosphoryl carrier in the enzyme. Subsequently, Kreil and Boyer (1964) identified phosphohistidine as the phosphoryl carrier in the E. coli enzyme. Recently, Cha, Cha and Parks (1965) also have suggested, based on electrophoretic separations of the pig heart enzyme, the possible

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occurrence of an activated form of succinic thiokinase, possibly a form containing enzyme-bound CoA. Almost simultaneously, using less purified enzyme fractions, Nishimura and Meister (1965) suggested that succinyl phosphate may participate in the overall succinic thiokinase reaction. This paper presents evidence consistent with the enzyme-CoA intermediate suggested by Upper (1964).

Materials.--^{32}P-labeled 3′-dephosphoCoA was prepared from 4′-phosphopantetheine and ^{32}P-AMP morpholidate as described by Moffatt and Khorana (1961). ^{32}P-AMP was synthesized from 2′,3′-isopropylidine adenosine and ^{32}P-cyanoethyl phosphate (Pfitzner and Moffatt, 1964) as outlined by Tener (1962). The morpholidate was prepared as outlined by Moffatt and Khorana (1961). Presumably because of the extremely high radioisotope content and as a result of scaling the reaction volume down, our yields ranged around 10 to 15 per cent. Unlabeled 3′-dephosphoCoA was prepared by the same procedure. For isolation, the 3′-dephosphoCoA was oxidized with I_2 to the disulfide and eluted from DEAE-cellulose, with a linear gradient of 0 to 0.7 M triethyl ammonium bicarbonate. All other substrates were obtained commercially.

The succinic thiokinase was purified from E. coli (Crooks strain) by the Upper procedure (Upper, 1964) and was the generous gift of Dr. Robert Ramaley. The enzyme had a specific activity > 15 μmoles of thioester formed/min/mg protein and was considered to be virtually homogeneous.

Results.--In order to make use of 3′-dephosphoCoA as a probe in the succinic thiokinase catalyzed reaction we first had to establish its reactivity as a substitute for CoA. The reciprocal plot shown in Fig. 1 gives a Michaelis constant for 3′-dephosphoCoA of approximately 10^{-4} M. The value for CoA with this enzyme is 5×10^{-6} M*. Although often added

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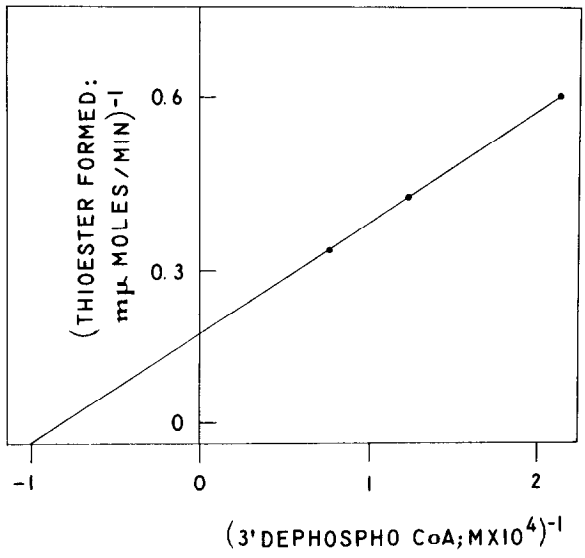

Fig. 1. Reciprocal plot, rate of thioester formation versus 3′-dephosphoCoA concentration. Reaction started by enzyme addition after 1 minute equilibration in Cary Model 11 cell compartment. Thioester formation followed at 232 mμ. Reaction mixture contained in mM concentration in 1 ml cuvettes: Tris buffer, pH 7.2, 50; $MgCl_2$, 10; 3′-dephosphoCoA, 0.047 to 0.144 as indicated; ATP, 0.3; succinate, 10; enzyme, 6.5 x 10^{-3} units (3.7 units/mg).

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to succinic thiokinase reactions, mercaptans such as cysteine or β-mercaptoethanol were, in our hands, slightly inhibitory (35% - 50% inhibition at 10 mM) and thus were avoided throughout this work. By using carefully prepared Sephadex G-50 columns, as shown in Fig. 2, it was possible to demonstrate association of ^{32}P-3′-dephosphoCoA with succinic thiokinase. Succinic thiokinase labeled with ^{32}P-3′-dephosphoCoA was prepared by incubating the enzyme first with ATP and $MgCl_2$ (5 μmoles each) in 0.5 ml of Tris-HCl pH 7.2 for 10 minutes at 25°. The reaction was stopped by adding 25 μmoles of EDTA, and the reaction mixture was passed through a 1 cm x 60 cm column of Sephadex G-50 which had been previously equilibrated with 0.05 M Tris-HCl (pH 7.2). The phosphorylated form of the enzyme thus collected was reincubated with $MgCl_2$ (25 μmoles) and ^{32}P-3′-dephosphoCoA (0.5 μmole; 12 x 10^5 cpm/μmole) in 3 ml of 0.05 M Tris .05 M KCl, pH 7.2 for 15 minutes at 25°. This reaction mixture

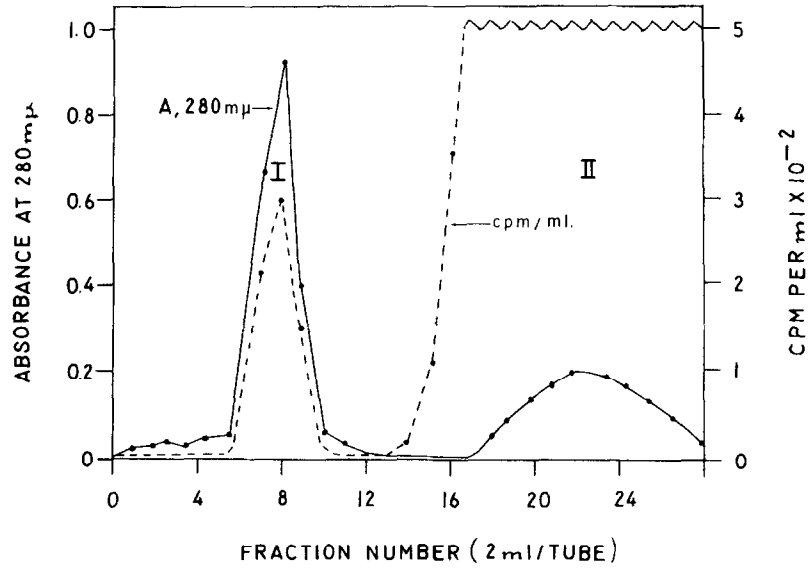

Fig. 2. Succinic thiokinase-dephosphoCoA isolation on Sephadex. Enzyme, 36 units (6 units/mg protein) pretreated as in the text, incubated with 0.5 μmole ^{32}P-3′-dephosphoCoA was separated from small molecules by passage through a 1 x 60 cm column of Sephadex G-50. Fraction volume, 2 ml.

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was passed over another 1 x 60 cm Sephadex G-50 column providing the elution pattern shown in Fig. 2. The three fractions containing protein showed ratios of 0.21, 0.2, and 0.21 moles of 3′-dephosphoCoA per mole of succinic thiokinase.

Evidence suggesting that the ^{32}P-3′-dephosphoCoA was covalently bound to the enzyme is presented in Table I. In this case the enzyme dephosphoCoA fractions previously obtained (Fig. 2) were stored in ice over several hours, during which some of the bound 3′-dephosphoCoA dissociated and the ratio of material bound per mole of enzyme dropped to around 0.13. In Experiment 2 (Table I) pooled succinic thiokinase fractions similarly treated contained 0.3 moles 3′-dephosphoCoA bound per mole enzyme. Following the treatments indicated in Table I some high molecular weight "Blue Dextran" was added as a marker and the reaction mixture passed over a 0.7 x 35 cm beaded Sephadex G-50 column

and eluted with 0.05 M Tris (pH 7.2) 0.05 M KCl. The protein and "Blue Dextran" eluted together and thus the entire colored fraction was collected and dried on a planchet for counting on a gas flow detector.

The results in Table I show that the enzyme bound 3′-dephosphoCoA is stable to base and strong acid but clearly labile to weak acid (pH 2 to 5).

TABLE I

Properties of the DephosphoCoA SuccinylCoA Synthetase Compound

Treatment, 25° for 5 minutes	Moles dePCoA/mole enzyme[a]	
	Expt. 1	Expt. 2
pH 7.2	0.13	0.50
pH 12	0.14	0.50
pH 2 (Expt. 1):pH 3 (Expt. 2)	0.07	0.36
pH < 1	0.12	-
Succinate; $MgCl_2$ (5 mM each)	0.12	0.36
" " CoA (0.5 mM)	0.04	-
" " CoA (0.2 mM)	-	0.27
$MgCl_2$ (5 mM); CoA (0.2 mM)	-	0.48
" SuccinylCoA (0.5 mM)	-	0.20
" P_i (50 mM)	-	0.45
" P_i (1 mM)	-	0.45
" " CoA (1.2 mM)	-	0.43
" " ADP (0.5 mM)	-	0.30
NH_2OH (50 mM) pH 7.2 Expt. 1; pH 10 Expt. 2	0.17	0.50
β-mercaptoethanol (50 mM, pH 7.2)	0.13	0.53
NH_2OH, " (50 mM each, pH 8); urea 4M	0.17	-
Bromine water (pH 1 Expt. 1; pH 8.0 Expt. 2)	0.05	0.21

Enzyme-dephosphoCoA was isolated on Sephadex G-50, 0.2 ml aliquots of the protein peak treated as indicated. Reaction mixtures, in the presence of "Blue Dextran" (Sephadex), were passed through 0.7 x 35 cm columns of Sephadex G-50 and the entire effluent of each sample dried on a planchet before counting on a gas flow counter. In Experiment 1 the enzyme used contained 6 units per mg of protein and in Experiment 2, 10.5 units of succinic thiokinase per mg protein.

[a] Computed on a M.W. of 160,000 for succinylCoA synthetase.

Treatment with bromine water is also effective in removing the labeled nucleotide. Succinate, or CoA alone (separate experiment) do not remove the labeled nucleotide. However, succinate together with CoA (catalytic level) is effective in causing removal of the bound nucleotide. The radioactive coenzyme discharged from the enzyme was recovered as succinyl dephosphoCoA together with 3′-dephosphoCoA as demonstrated by paper electrophoresis at pH 3.5 and by ion exchange chromatography on Dowex-1 with a formic acid ammonium formate gradient by the procedure of Cha and Parks (1964). ^{32}P-AMP was not found in the reactions. Urea, NH_2OH or β-mercaptoethanol alone or in combination are ineffective in removing the dephosphoCoA from the protein. These results suggest that neither a thiolester nor a disulfide linkage can account for the observed dephosphoCoA binding.

The labeled coenzyme is removed chemically under conditions similar to those required for cleavage of a thiolphosphate (Akerfeldt, 1960), (Herr and Koshland, 1957). However, evidence for the presence of phosphate other than phosphohistidine in succinic thiokinase has not been obtained.

These experiments are consistent with the formation of a covalently linked enzyme dephosphoCoA compound in the reaction catalyzed by succinic thiokinase, but by no means establish it as an obligatory intermediate. Further careful kinetic examination and evaluation of the system will be required to establish the validity of this possibility. In all of our experiments, enzyme has been used in stoichiometric amounts and thus sluggish side reactions could be magnified and could account for our observations. However, the fact that all the partial reactions observed here and by others (Upper, 1964), (Kreil and Boyer, 1965) and (Cha, Cha and Parks, 1965) are slow relative to the rate of the overall reaction may be interpreted as an explicit requirement by this enzyme for all of the reaction components in order to achieve the most catalytically active conformation. Thus in the removal of dephosphoCoA by succinate the addition of CoA showed

a stimulatory effect and similarly in the discharge of dephosphoCoA with P_i, ADP showed an effect. The latter also could have resulted from a favorable equilibrium toward ATP formation.

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