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The Mechanism of the Succinic Thiokinase Reaction. Effector Role of Desulfo-coenzyme A in Succinyl Phosphate Formation*

Frederick Lawrence Grinnell and Jonathan S. Nishimura

ABSTRACT: Incubation of highly purified *Escherichia coli* succinic thiokinase (succinate: coenzyme A ligase (adenosine diphosphate), EC 6.2.1.5) and the coenzyme A analog, desulfo-coenzyme A, with succinate, adenosine triphosphate, and Mg^{2+} resulted in stimulation of succinyl phosphate synthesis. This suggests that coenzyme A is required for optimal formation of enzyme-bound succinyl phosphate. The K_m of desulfo-coenzyme A in this reaction was 6.2×10^{-5} M. A virtually absent ATPase activity of the enzyme was significantly stimulated by both coenzyme A and desulfo-coenzyme A. The K_m of desulfo-coenzyme A in this reaction was 1.4×10^{-3} M. The possible significance of these results is discussed. During these studies it was found that succinyl phosphate is converted nonenzymatically into succinamic acid in the presence of $(NH_4)_2SO_4$ at pH 7.2.

It has been postulated that optimal formation of enzyme-bound succinyl phosphate by succinic thiokinase may require the presence of CoA[1] (Nishimura, 1967). This suggestion was based on the observation that succinate reacted with phosphorylated succinic thiokinase to form succinyl phosphate, but at a slow rate. However, this hypothesis was difficult to test directly, as virtually no succinyl phosphate was detectable in the enzymatic system when CoA was present. It seemed reasonable, therefore, to investigate the use of CoA analogs as possible effectors of succinyl phosphate synthesis by succinic thiokinase. Desulfo-CoA (Chase *et al.*, 1966) was chosen for this study because it possesses all the primary structural features of CoA but lacks a sulfhydryl group. It had also been demonstrated that desulfo-CoA interacted with several CoA enzymes and was relatively easy to prepare (Chase *et al.*, 1966).

The results described in this paper show that desulfo-CoA stimulates the synthesis of succinyl phosphate by reaction of ATP, Mg^{2+}, and succinate with highly purified preparations of *Escherichia coli* succinic thiokinase, which strongly suggests the formation of an enzyme–CoA–succinyl phosphate complex in the catalytic reaction. In addition, a second binding site for CoA on the enzyme has been deduced. The significance of this binding site and its possible relationship to a high-energy nonphosphorylated form of the enzyme (Cha *et al.*, 1965, 1967a,b; Moyer *et al.*, 1967) is discussed.

Experimental Section

Materials. ATP and ADP were purchased from Sigma. CoA (lithium salt) was purchased from P-L Biochemicals. Sephadex G-10 was obtained from Pharmacia Fine Chemicals and prepared according to the manufacturer's recommendations. [^{32}P]Phosphate was obtained from New England Nuclear Corp. as were [2,3-^{14}C]succinic acid and [^{14}C]*p*-mercuribenzoate. [γ-^{32}P]ATP was synthesized enzymatically (Glynn and Chappell, 1964) and purified as described in the preceding communication (Grinnell and Nishimura, 1969).

Enzyme. Succinic thiokinase was isolated and assayed as described in the preceding paper (Grinnell and Nishimura, 1969).

Electrophoretic Separations and Radioactivity Measurements. Separation of reaction products was effected by paper electrophoresis at pH 7.4 (Cha *et al.*, 1967b). Aliquots of the reaction solutions (10–20 μl) were spotted on 75 × 2.54 cm strips of moistened Whatman 3MM paper and a potential of 20V/cm was applied for 90 min at

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[1] Abbreviations used: CoA, coenzyme A; CoAH, desulfo-coenzyme A.

0°. The strips were dried and counted in a Nuclear-Chicago Actigraph III strip scanner equipped with scaler. Typical distances moved by various compounds in centimeters, from the origin, were as follows: ATP, 15.0; P_i, 22; succinyl hydroxamate, 16.0; succinamate, 16.5; succinyl-CoA, 17; succinyl phosphate, 23; and succinate 27.

Electrophoretic separation was also carried out at pH 3.6 (Sato *et al.*, 1963). The potential applied was 53 V/cm over 75 × 2.54 cm strips of Whatman No. 3MM for 1 hr at 0°. In this system typical distances moved, in centimeters from the origin, were: succinamate or succinyl hydroxamate, 7.5; succinate, 14; and succinyl-CoA, 32.

When samples were analyzed by scintillation counting, they were mixed with a naphthalene–dioxane solution (Bray, 1960) and counted in a Nuclear-Chicago liquid scintillation spectrophotometer.

Desulfo-coenzyme A Preparation. Desulfo-coenzyme A was prepared according to the method of Chase *et al.* (1966). CoA (100 mg) was treated with 1 g of activated Raney nickel catalyst at pH 5.1, 37°, in a reaction mixture containing 0.4 mmole of ammonium acetate and 0.5 ml of saturated EDTA in a final volume of 6.5 ml. The reaction was run to completion (75 min) as determined by analysis of 10 μl aliquots with 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman, 1959). The supernatant, after low-speed centrifugation, was allowed to sit overnight at 0°. This was brought to 10 ml with distilled water and 0.6 ml of 30% hydrogen peroxide was added. After 20 min the solution was diluted to 35 ml and placed on a DEAE-cellulose (Cl-form) column (40 × 4 cm) and eluted with a linear chloride gradient established with 2.0 l. of 0.003 N HCl and 2.0 l. of 0.003 N HCl–0.2 M LiCl as described by Moffatt and Khorana (1961). Two peaks, as judged by their adsorption at 260 mμ, were obtained, one of which contained oxidized CoA, and the other, desulfo-coenzyme A. The latter accounted for 66% of the starting material. This fraction was concentrated by flash evaporation and desalted on Sephadex G-10 (1.5 × 20 cm column equilibrated with water). Salt-free fractions containing the final product were evaporated to dryness and stored at −20°.

Amino Acid Analysis. Amino acid analyses of hydrolysates of CoA and desulfo-CoA were carried out in a Phoenix Model 6800 automated amino acid analyzer.

Results

Purity of Desulfo-CoA. Desulfo-CoA was prepared as described in the Experimental Section. Desulfo-CoA (1.06 μmoles) and CoA (1.04 μmoles) were subjected to conditions of performic acid oxidation (Hirs, 1956) and then hydrolyzed in 6 N HCl for 16 hr at 100°. The results of amino acid analysis and phosphate analysis (Gomori, 1942) of the hydrolysates are shown in Table I. The absence of taurine and presence of ethylamine in the desulfo-CoA hydrolysate were anticipated.

Desulfo-CoA was also examined for possible contamination by small quantities of CoA. Desulfo-CoA (0.53 μmole) was mixed with *p*-mercuribenzoate (0.19 μmole; 2.5 μCi/μmole), according to the method of Boyer (1954).

TABLE I: Component Analysis of Desulfo-CoA and CoA.[a]

Component Analyzed	Moles/Mole of Adenine[b]	
	CoAH	CoA
Phosphate	3.30	3.10
β-Alanine	1.09	0.94
Taurine	None	0.96
Ethylamine	0.93	None

[a] See text for explanation. [b] Adenine content by A_{260} on sample prior to oxidative hydrolysis.

The reaction mixture was passed through a Sephadex G-10 column which separated desulfo-CoA, CoA, and CoA–*p*-mercuribenzoate from *p*-mercuribenzoate. Suitable controls were run (minus desulfo-CoA; plus CoA) and it was found that a CoA contaminant of less than 0.01% was present.

Inhibition of the Succinic Thiokinase Reaction by Desulfo-CoA. Desulfo-CoA was found to inhibit succinic thiokinase activity in an adaptation of the hydroxamate assay (Kaufman *et al.*, 1953), as shown in Table II. The results indicate that significant inhibition occurred when the analog was present at a concentration approximately 30 times that of CoA. Thorough kinetic studies of this inhibition have not been practically possible, owing to the quantity of desulfo-CoA needed in the conventional assay procedures. However, the inhibition appears to be of the competitive type. Chase *et al.* (1966) have reported that all of the five CoA enzymes which they found were affected by desulfo-CoA were inhibited competitively.

Stimulation of Succinyl Phosphate Formation by Desulfo-CoA. When desulfo-CoA was added to incubations containing enzyme, ATP, and Mg^{2+}, succinyl phosphate

TABLE II: Inhibition of Succinic Thiokinase by Desulfo-CoA.[a]

CoAH (mμmoles)	Succinyl Hydroxamate (mμmoles/5 min per unit)	% Inhibn
None	17.5	0
10.0	14.0	20
25.0	10.8	38

[a] The reaction mixtures contained enzyme (0.02 unit; specific activity 580), Tris-HCl (2 μmoles; pH 7.2), $MgCl_2$ (0.2 μmole), ATP (0.2 μmole), CoA (0.78 mμmole), dithiothreitol (1.8 mμmoles), [2,3-^{14}C]-succinate (0.08 μmole; 4.5 μCi/μmole), neutralized NH_2OH (20 μmoles), and CoAH as indicated, in a final volume of 0.04 ml. After incubation at 37° for 5 min, the reaction was terminated by rapid freezing in a Dry Ice–acetone bath (−60°). Aliquots of the thawed solutions were subjected to paper electrophoresis and analyzed, as described in the Experimental Section.

synthesis was observed. The results are shown in Figure 1. The data clearly demonstrate that desulfo-CoA is required for succinyl phosphate synthesis, under these conditions. A surprising observation made was the appearance of another succinyl compound, succinamate (succinic acid monoamide), which was subsequently identified and shown to arise from the nonenzymatic reaction of succinyl phosphate and ammonia, present in the enzyme preparations (Grinnell and Nishimura, 1969). The proof of structure of succinamate will be discussed later in the text.

Analysis of the kinetic relationships between the curve in Figure 1 reveals that a product–precursor correlation exists between succinamate and succinyl phosphate. This can be described by eq 1, where (succinyl phosphate)

$$\text{d(succinyl phosphate)}/dt = [V - k(\text{succinyl phosphate})] \quad (1)$$

is the apparent succinyl phosphate concentration, V is the actual velocity of succinyl phosphate formation (which is zero order under the initial velocity conditions reported here), and k is the pseudo-first-order rate constant for succinamate synthesis. When the expression in brackets is equal to zero, at some succinyl phosphate concentration, the plateau in the time-dependence curve appears.

Some variation in the ratio of succinyl phosphate to succinamate was observed in different experiments. This is probably a function of the ammonia concentration in the incubation mixture which would influence k, as will be noted later in the text. Succinyl derivative (succinyl phosphate plus succinamate) formation is expected to be related to the concentration of enzyme, ATP, succinate, and desulfo-CoA in the incubations. This is what has been observed.

The Effect of Enzyme Concentration on Succinyl Derivative Synthesis in the Presence of Desulfo-CoA. That there is an approximately linear relationship between enzyme and succinyl derivative (succinyl phosphate and succinamate) synthesis is shown in Figure 2. The values of product obtained at lower enzyme concentrations are subject to much greater error than those at higher concentrations of enzyme. The fact that the plotted points lie beneath the lines at lower enzyme concentrations is probably indicative of this.

The Effect of Succinate and ATP Concentrations on Succinyl Derivative Synthesis in the Presence of Desulfo-CoA. Succinyl phosphate and succinamate formation are directly proportional to the amount of succinate added to the incubation up to concentrations of 20 mM (higher concentrations have not been tested) under the reaction conditions shown for Figure 1. This is surprising and implies that the apparent K_m for succinate in this reaction must be rather high. However, it is fair to point out that what we may be measuring is not the rate of succinyl phosphate synthesis but the dissociation of bound succinyl phosphate from the enzyme. This possibility makes interpretation of the kinetic data more difficult.

The dependence of succinyl derivative formation on ATP concentration was also measured (at 2 mM succinate) and the half-saturating concentration was no more than 0.25 mM. Furthermore, ADP is shown to be an inhibitor of the reaction (Table III), which one would expect if phosphorylated enzyme is an intermediate.

The Effect of Desulfo-CoA on Succinyl Phosphate Synthesis. As illustrated in Figure 3, desulfo-CoA stimulates succinyl phosphate formation at very low concentrations. The K_m for desulfo-CoA by the double-reciprocal plot method (Lineweaver and Burk, 1934) gave a value of 6.2×10^{-5} M. As expected from the kinetic evalua-

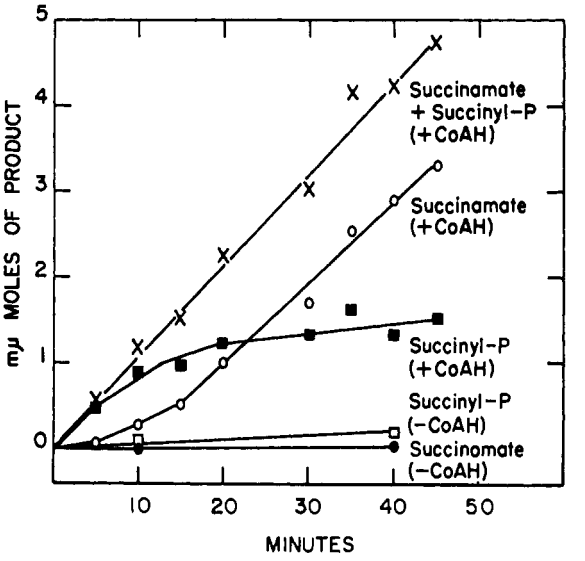


FIGURE 1: Time dependence of succinyl phosphate and succinamic acid formation, in the presence and absence of desulfo-CoA. The reaction mixtures contained enzyme (8 units; specific activity 500), Tris-HCl (1 μmole; pH 7.2), ATP (0.1 μmole), $MgCl_2$ (0.1 μmole), CoAH (0.52 μmole), and [2,3-^{14}C]succinate (0.04 μmole; 4.5 μCi/μmole) in a final volume of 0.02 ml. After incubation at 37° for the times indicated, the reactions were stopped by freezing. Aliquots of the thawed solutions were electrophoresed and analyzed as described in the Experimental Section.

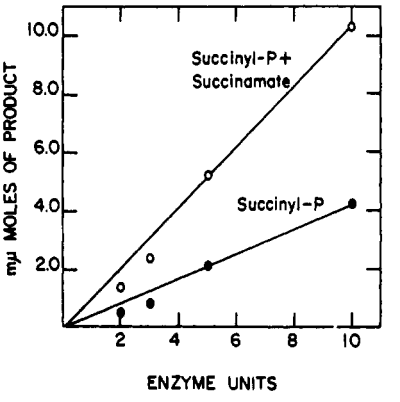


FIGURE 2: Enzyme dependence of succinyl phosphate and succinamic acid formation. The reaction mixtures contained Tris-HCl (1 μmole; pH 7.2), ATP (0.1 μmole), $MgCl_2$ (0.1 μmole), [2,3-^{14}C]succinate (0.04 μmole; 4.5 μCi/μmole), CoAH (0.052 μmole), and enzyme, as indicated, in a final volume of 0.02 ml. After incubation at 37° for 35 min, the reactions were stopped by freezing. Aliquots of the thawed solutions were electrophoresed and analyzed as described in the Experimental Section.

TABLE III: Inhibition of Succinyl Phosphate and Succinamic Acid Formation by ADP.[a]

ADP (mμmoles added)	Succinyl-P (mμmoles formed)	Succinyl-P and Succinamate (mμmoles formed)	Succinyl-P (% inhibn)	Succinyl-P and Succinamate (% inhibn)
0	2.37	3.97	0	0
20	1.76	3.01	25.7	24.2
100	0.94	1.62	60.3	58.7

[a] The reaction mixtures contained enzyme (6.5 units; specific activity 580), Tris-HCl (2 μmoles; pH 7.2), ATP (0.2 μmole), $MgCl_2$ (0.2 μmole), [2,3-^{14}C]succinate (0.08 μmole; 4.5 μCi/μmole), and ADP, as indicated, in a final volume of 0.04 ml. After incubation for 35 min at 37°, the reaction was stopped by freezing. Aliquots of the thawed solutions were electrophoresed and analyzed as described in the Experimental Section.

tion presented earlier, this value is independent of whether apparent succinyl phosphate or total succinyl derivative is measured.

Establishment of the Identity of Succinamic Acid and Its Formation from Succinyl Phosphate and Ammonia. The surprising appearance of a succinyl compound in addition to succinyl phosphate led us to examine the relationship between the two compounds. The data in Table IV show that not only could the unknown compound be formed nonenzymatically, but that it also arose directly from succinyl phosphate. When a reaction mixture which contained 1.3 mμmoles of succinyl phosphate and 2.48 mμmoles of the unknown was heated, succinyl phosphate disappeared and 3.64 mμmoles of the unknown was found. Then, when nonradioactive succinate and additional enzyme were added to a reaction mixture in which 1.74 mμmoles of succinyl phosphate and 3.52 mμmoles of the unknown were present and the mixture was allowed to incubate further, much more labeled unknown was formed than would have been expected if it had been synthesized directly from the diluted [^{14}C]-

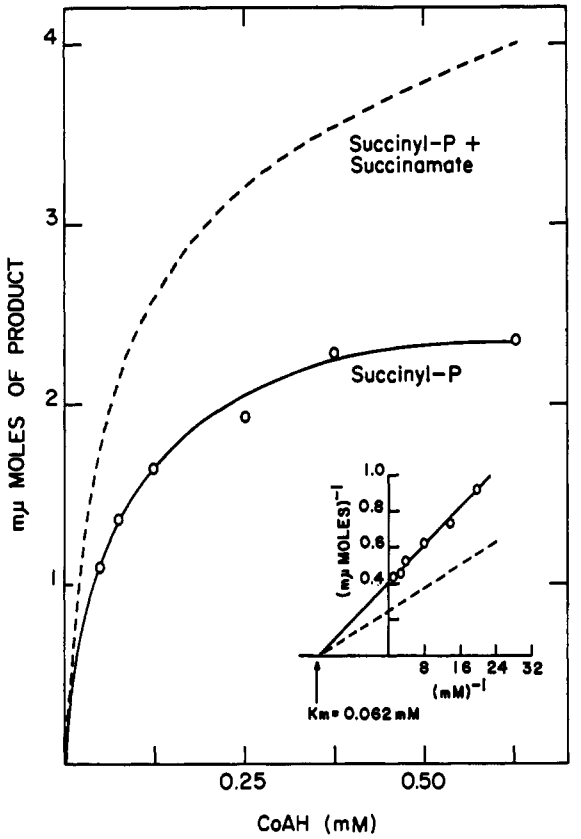


FIGURE 3: Desulfo-CoA dependence of succinyl phosphate formation. The reaction mixtures contained enzyme (13 units; specific activity 580), Tris-HCl (2 μmoles; pH 7.2), ATP (0.2 μmole), $MgCl_2$ (0.2 μmole), [2,3-^{14}C]succinate (0.08 μmole; 4.5 μCi/μmole), and CoAH as indicated, in a final volume of 0.04 ml. After incubation at 37° for 35 min, the reactions were stopped by freezing. Aliquots of the thawed solutions were electrophoresed and analyzed as described in the Experimental Section.

TABLE IV: Conversion of Succinyl Phosphate into Succinamic Acid.[a]

Expt	Treatment	Succinyl-P (mμmoles)	Succinamate (mμmoles)
1	None	1.30	2.48
2	Heated[b]	None[c]	3.64
3	None[d]	1.74	3.52
4	Diluted with unlabeled succinate[e]	0.23	5.15

[a] The reaction mixtures contained enzyme (7 units; specific activity 500), Tris-HCl (1 μmole; pH 7.2), ATP (0.1 μmole), $MgCl_2$ (0.1 μmole), CoAH (0.078 μmole), and [2,3-^{14}C]succinate (0.04 μmole; 4.5 μCi/μmole) in a final volume of 0.02 ml. After incubation for 35 min at 37°, the reaction was stopped by freezing. Aliquots of the thawed solutions were electrophoresed and analyzed as described in the Experimental Section. [b] An aliquot from expt 1 (*ca.* 10 μl), which had been kept frozen overnight, was placed in a boiling water bath for 5 min. Water (15 μl) was added and an aliquot of this solution was electrophoresed and analyzed as described. [c] There was no change in a nontreated control which had been kept frozen overnight. [d] Same as expt 1, except that 0.052 μmole of CoAH was added. [e] Unlabeled succinate (1 μmole) and enzyme (0.7 unit) were added to a 10-μl aliquot of expt 3, which had been kept frozen overnight. The final volume was 0.012 ml. The solution was then electrophoresed and analyzed as described in the Experiment Section.

succinate. Therefore, the unknown compound was synthesized directly from a precursor (succinyl phosphate) which was present before the unlabeled succinate was added.

The results described in Table V show that the succinyl compound in question was an anion and had no apparent cationic properties. Its adsorption on and elution (at 0.4 N HCOOH) from Dowex 1 were quite similar to the behavior of succinyl hydroxamate on this ion exchanger. The fact that the compound was hydrolyzed upon treatment with 2 N HCl (100° for 2 hr) and that it was resistant to saponification (1 N KOH at room temperature for 1 hr) suggested an amide-like linkage and not an ester. Attention was therefore focused on the possibility that the unknown compound was succinamic acid, formed by the reaction of succinyl phosphate with ammonia in the enzyme preparations. This was confirmed by experiments with chemically synthesized [^{14}C]-succinyl phosphate (Nishimura and Meister, 1965). Treatment of the compound with 7 M NH_4OH gave quantitative conversion to a compound which had the same properties as the unknown. Similar treatment of [^{14}C]-succinic acid monobenzyl ester overnight at room temperature gave the same result. In addition, reaction of chemically synthesized [^{14}C]succinyl phosphate under incubation conditions, with and without enzyme, and in the presence of varying concentrations of $(NH_4)_2SO_4$ (0–1.8 M) showed that succinamic acid was formed from succinyl phosphate and that this formation was independent of enzyme, but dependent upon ammonium ion (presumably the uncharged NH_3 form). In the absence of ammonium sulfate succinyl phosphate was hydrolyzed. However, when $(NH_4)_2SO_4$ was added, succinamic acid formation was favored. A concentration of 0.25 M $(NH_4)_2SO_4$ was sufficient to almost completely prevent hydrolysis of succinyl phosphate. The rate of succinamic acid formation increased slightly as the $(NH_4)_2SO_4$ concentration was raised. As pointed out in the accompanying paper (Grinnell and Nishimura, 1969), the enzyme preparations are maintained in $(NH_4)_2SO_4$ solution to ensure stability. Thus, $(NH_4)_2SO_4$ was present in our experiments from the addition of the enzyme solution. Some effect on the apparent rate constant for nonenzymatic formation of succinamate from succinyl phosphate, k (see eq 1), is expected as one varies $(NH_4)_2SO_4$ concentration, but it does not appear that total succinyl phosphate synthesis (succinamate plus succinyl phosphate) is significantly affected.

A similar reaction between ammonia and succinyl-CoA has been observed in this laboratory. This is of obvious importance in the assay of the enzyme, particularly if the formation of succinyl-CoA is measured. We have found that $(NH_4)_2SO_4$ has little effect on the assay procedure employed in this laboratory (Kaufman, 1955), evidently because hydroxylamine is virtually in the uncharged form at pH 7.4 and, at 0.5 M NH_2OH and comparatively lower $(NH_4)_2SO_4$ concentrations, formation of the hydroxamate should be favored over that of the amide.

Stimulation of ATPase Activity by Desulfo-CoA and CoA. In a previous report from this laboratory (Grinnell and Nishimura, 1969) it was observed that highly purified succinic thiokinase preparations were virtually void of ATPase activity. The results illustrated in Figure 4 compare the effects of CoA and desulfo-CoA in stimulating ATPase activity. Desulfo-CoA was more effective in this respect than CoA. This interesting finding indicates that this activation is probably one which requires most of the primary structure of CoA, but not the sulfhydryl group. The effect of desulfo-CoA concentration on this phenomenon is shown in Figure 5. By the double-reciprocal plot method (Lineweaver and Burk, 1934) a K_m for desulfo-CoA of 1.4 mM was obtained, which is over 20-fold higher than the K_m for desulfo-CoA in succinyl phosphate formation.

TABLE V: Dowex Chromatography of Succinamic Acid.[a]

Column	Eluting Agent (ml)	% Radioactivity of sample[b]
Dowex 1 $HCOO^-$ (1 × 2 cm)	H_2O (10)	0.2
	0.4 N HCOOH (5)	12.0[c]
	0.4 N HCOOH (5)	1.3
	4.0 N HCOOH (5)	86.5[d]
Dowex 50 H^+ (1 × 2 cm)	H_2O (10)	99.6
	2.0 M NH_4OH (5)	0.4

[a] The reaction mixture contained enzyme (20 units; specific activity 580), Tris-HCl (25 μmoles; pH 7.2), ATP (2.5 μmoles), $MgCl_2$ (2.5 μmoles), CoAH (0.625 μmole), and [2,3-^{14}C]succinate (0.5 μmole; 4.5 μCi/μmole) in a final volume of 0.2 ml. After 2 hr at 37°, the reaction was stopped by freezing. [b] Prior to column treatment, distribution of radioactivity was: succinate, 86%; succinyl phosphate, 0.7%; and succinamic acid, 11%. [c] An aliquot was concentrated by lyophilization and redissolved in 0.02 ml of H_2O. This was electrophoresed and analyzed as described in the Experimental Section. Nearly all of the radioactivity migrated in the succinamic acid area. [d] Succinic acid eluted in this fraction.

Discussion

The data in this paper indicate that succinyl phosphate is rapidly formed and released by succinic thiokinase, in the absence of CoA, when desulfo-CoA is added to incubation mixtures. This reaction is dependent upon the concentrations of enzyme, succinate, ATP, and desulfo-CoA. Earlier demonstrations of succinyl phosphate formation in the absence of CoA (Nishimura and Meister, 1965; Nishimura, 1967) required much larger quantities of enzyme and higher succinate concentration, or both. The Michaelis constant for desulfo-CoA in the above reaction is 6.2×10^{-5} M. This is indicative of a tightly bound enzyme–desulfo-CoA complex. A second observation made in this report is that desulfo-CoA activates an ATPase activity of the enzyme. The K_m for desulfo-CoA in this reaction was 1.4×10^{-3}

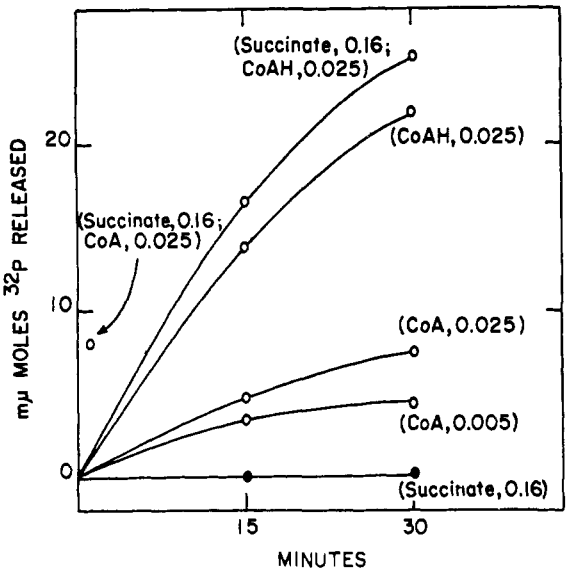


FIGURE 4: CoA and desulfo-CoA-dependent ATPase activity. The reaction mixtures contained enzyme (6 units, specific activity 410), Tris-HCl (2 μmoles), pH 7.2, [γ-^{32}P]ATP (0.2 μmole; 58,600 cpm), $MgCl_2$ (0.2 μmole), and CoA, CoAH, and succinate, as indicated (in micromoles), in a final volume of 0.04 ml. The CoA contained a 2-fold molar excess of dithiothreitol. After incubation at 37° for the specified times, the reaction was stopped by freezing. Aliquots of the thawed solutions were electrophoresed and analyzed as described in the Experimental Section.

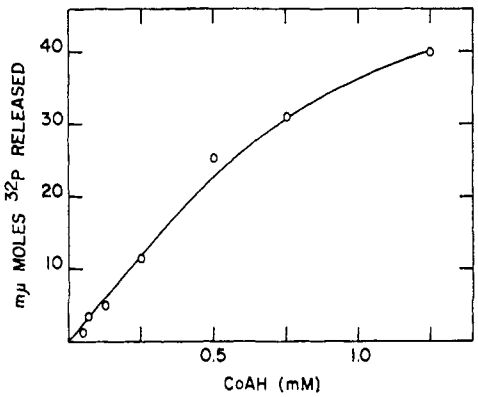
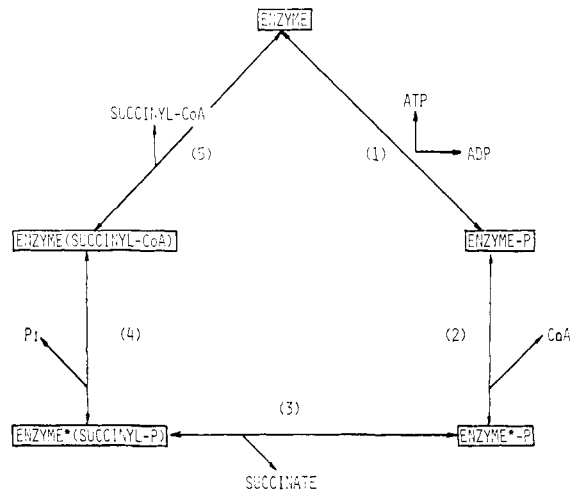


FIGURE 5: Desulfo-CoA dependence of the ATPase reaction. The reaction mixtures contained enyzme (6 units; specific activity 410), Tris-HCl (2 μmoles; pH 7.2), [γ-^{32}P]ATP (0.2 μmole, 58,000 cpm), $MgCl_2$ (0.2 μmole), succinate (0.08 μmole), and CoAH as indicated, in a final volume of 0.04 ml. After incubation for 30 min at 37°, the reactions were stopped by freezing. Aliquots of the thawed solutions were electrophoresed and analyzed as described in the Experimental Section.

M, more than 20 times higher than that in succinyl phosphate formation. Furthermore, desulfo-CoA is actually more effective than CoA itself in stimulating ATPase. The formation of succinyl phosphate is considered to further support the proposed role of this compound in the mechanism of succinic thiokinase (Nishimura and Meister, 1965; Nishimura, 1967). The desulfo-CoA-induced ATPase activity and desulfo-CoA-stimulated succinyl phosphate formation seem to occur simultaneously. This implies multiple sites for CoA with the enzyme. The differences in the Michaelis constants for desulfo-CoA in these reactions lend support to this contention.

Desulfo-CoA stimulation of succinyl phosphate formation suggests that reaction of succinate with phosphorylated enzyme requires CoA. Succinyl phosphate which is formed in the presence of desulfo-CoA then dissociates from the enzyme, since the analog is nonreactive. It is apparent that the experiments described here do not necessarily reflect the actual rate at which enzyme-bound succinyl phosphate is formed. Determination of this rate will require further investigation. The ability of CoA to stimulate reactions associated with the thiokinase has been observed elsewhere. In the previous communication (Grinnell and Nishimura, 1969) ATP $\rightleftarrows$ ADP exchange was found to be dependent upon CoA, and Ramaley *et al.* (1967) have reported enhanced rates of enzyme phosphorylation with CoA addition at low temperatures. The simplest explanation for these observations is that there exists an activated succinic thiokinase form which contains bound CoA. The indications are, however, that this is a noncovalent complex. This form of the enzyme has been designated "enzyme*" in Scheme I.

SCHEME I



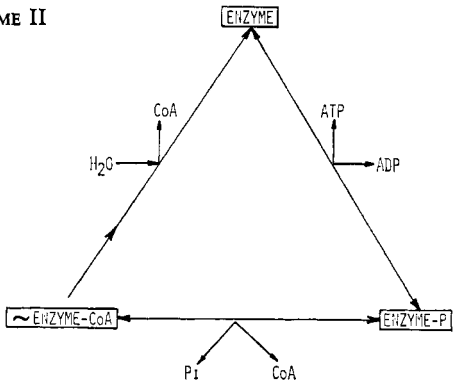
Scheme I illustrates in highly simplified form the proposed succinyl phosphate mechanism. Of singular importance is the observation made by Moyer *et al.* (1967) that oxygen is probably transferred directly from succinate to P_i. Furthermore, Cha *et al.* (1967b) have presented good evidence for succinyl-CoA binding to the enzyme, and the requirement of P_i in the succinate $\rightleftarrows$ succinyl-CoA exchange reaction (Grinnell and Nishimura, 1969) is clearly indicated by this mechanism (reactions 3–5).

We are presently unclear on the precise nature of reactions 1 and 2 in Scheme I. Although CoA is required for ATP $\rightleftarrows$ ADP exchange to occur, it is not required for phosphorylation of the enzyme (Grinnell and Nishimura, 1969; Upper, 1964). However, it has been clearly shown that ADP is not bound in significant amount to the phosphorylated enzyme (Upper, 1964; Nishimura, 1967; Cha *et al.*, 1965, 1967a; Moyer *et al.*, 1967). Studies on the interactions of desulfo-CoA with the phosphorylated enzyme, currently in progress in this lab-

oratory, may be able to resolve this question. It would be very interesting to know whether the CoA which stimulates ATP ⇄ ADP exchange is the same CoA that ultimately reacts with succinyl phosphate to form succinyl-CoA.

Due consideration should be accorded the evidence for a high-energy nonphosphorylated form of the enzyme. This hypothesis was based on the observation made by Upper (1964) that incubation of ^{32}P-phosphorylated succinic thiokinase with CoA yielded significant release of [^{32}P]P_i. Subsequently, Cha *et al.* (1965) showed that [^{3}H]CoA effected a release of [^{32}P]P_i with a simultaneous binding of an apparent stoichiometric amount of CoA to the enzyme. A similar demonstration of this phenomenon was made by Moyer and Smith (1966) who used [^{32}P]dephospho-CoA. Moyer *et al.* (1967) confirmed these findings, showing by spectrophotometric methods that release of [^{32}P]P_i by CoA from phosphorylated succinic thiokinase resulted in binding of CoA to the enzyme. However, in none of the experiments just summarized did a single subsequent incubation of the nonphosphorylated enzyme with succinate yield more than 20% of the expected amount of succinyl-CoA. That this discrepancy was not for the most part due to inactivation of the enzyme was clearly described by Moyer *et al.* (1967). A proposal which takes into account most of the known facts and experimental problems in this area, and which attempts to explain evidence which is not consistent with the succinyl phosphate pathway, is illustrated in Scheme II.

SCHEME II



This scheme postulates that CoA- (or desulfo-CoA-) stimulated ATPase is a manifestation of the slow hydrolysis of the high-energy nonphosphorylated form of the enzyme, in the anhydro form suggested by Moyer *et al.* (1967). It is suggested that a desulfo-CoA anhydro form of the enzyme is less stable and more susceptible to hydrolysis. Although Ramaley *et al.* (1967) have reported that an endogenous ATPase in their purified enzyme preparations is inhibited by CoA, Cha *et al.* (1967a) have cited a CoA-stimulated GTPase catalyzed by the pig heart enzyme. The scheme explains the CoA-dependent arsenolysis of nucleoside triphosphate (Cha *et al.*, 1967a) and the CoA-dependent ATP ⇄ P_i and P_i ⇄ phosphoryl enzyme exchange reactions (Cha *et al.*, 1967b; Ramaley *et al.*, 1967).

The fact that some succinyl-CoA may be formed when succinate is incubated with enzyme–CoA may be due to the presence of trace amounts of P_i and the resulting reformation of enzyme-P, followed by reaction of the latter with bound CoA and succinate (see Scheme I) to yield succinyl-CoA. That contamination by P_i may be a critical problem has been pointed out by Cha *et al.* (1967b) and is indicated in the sensitivity of the succinate ⇄ succinyl-CoA exchange reaction to P_i (Grinnell and Nishimura, 1969).

Schemes I and II are most likely interdependent pathways. However, it is not immediately obvious how they would interact. The kinetic binding data probably mean that Scheme I is most operative at very low CoA concentrations. The very existence of a high-energy nonphosphorylated form of the enzyme evokes great interest aside from its possible role as a catalytic intermediate. The suggestion that it may be a manifestation of a control mechanism (Moyer *et al.*, 1967) is pertinent in view of the fact that the enzyme performs a key function in the organism. This idea merits serious consideration.

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