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Succinic Thiokinase of *Escherichia coli*. Purification, Phosphorylation of the Enzyme, and Exchange Reactions Catalyzed by the Enzyme*

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ABSTRACT: Succinic thiokinase (succinate:coenzyme A ligase (adenosine diphosphate, EC 6.2.1.5) has been isolated in highly purified form from *Escherichia coli* (ATCC 4157) by a procedure which includes DEAE-cellulose chromatography and gel filtration on Sephadex G-150. Assuming a molecular weight of 141,000 (Ramaley, R. F., Bridger, W. A., Moyer, R. W., and Boyer, P. D. (1967), *J. Biol. Chem. 242* (4287), close to two phosphoryl groups are incorporated per mole of enzyme, whether the phosphorylating agent is adenosine triphosphate or inorganic phosphate (in the presence of succinyl-coenzyme A). Capability for phosphorylation appears to be related to enzyme activity and extent of phosphorylation by adenosine triphosphate is not significantly affected by coenzyme A at 2.5×10^{-5} M. At this concentration coenzyme A strongly stimulates the adenosine triphosphate $\rightleftarrows$ adenosine diphosphate exchange reaction catalyzed by the enzyme. It has been concluded that under these conditions coenzyme A is not bound covalently or involved in a high-energy nonphosphorylated form of the enzyme. It has also been found that inorganic phosphate is an almost complete requirement for the succinate $\rightleftarrows$ succinyl coenzyme A exchange reaction catalyzed by the enzyme, adding support to the hypothesis that enzyme-bound succinyl phosphate is an intermediate in the over-all reaction.

Previous publications from this laboratory (Nishimura and Meister, 1965; Nishimura, 1967) described experiments with partially purified preparations of succinic thiokinase from *Escherichia coli*, which provided evidence for enzyme-bound succinyl phosphate as an intermediate in the reaction catalyzed by this enzyme. Inferred from these experiments was a mechanism which can be summarized as follows:

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

$$\text{enzyme-P} + \text{succinate} \rightleftarrows \text{enzyme–succinyl-P} \quad (2)$$

$$\text{enzyme–succinyl-P} + \text{CoA} \rightleftarrows \text{enzyme} + \text{succinyl-CoA} + P_i \quad (3)$$

$$\text{ATP} + \text{succinate} + \text{CoA} \rightleftarrows \text{ADP} + \text{succinyl-CoA} + P_i \quad (4)$$

Reaction 1 has been demonstrated and studied in detail by several laboratories (Upper, 1964; Cha *et al.*, 1965; Nishimura, 1967; Ramaley *et al.*, 1967; Moyer *et al.*, 1967) and the site of phosphorylation has been shown to be the N-3 position of a histidine residue of the enzyme protein (Hultquist *et al.*, 1966). Evidence for reactions 2 and 3 was based on the observation that chemically synthesized succinyl phosphate gave rise to succinyl-CoA when incubated with the enzyme, Mg^{2+}, and CoA, and to enzyme-P when incubated with enzyme and Mg^{2+} (Nishimura and Meister, 1965). Reaction 2 was subsequently demonstrated directly (Nishimura, 1967). However, the release of succinyl phosphate from the enzyme was slow compared with the release of P_i from enzyme-P in the presence of succinate and CoA. For this reason it was suggested that CoA might be required to facilitate formation of the enzyme–succinyl phosphate complex. It was, therefore, desirable to obtain more information concerning the possibility of such a role of CoA and evidence that the succinyl phosphate pathway represents the major catalytic route of the succinic thiokinase reaction.

In the present investigation succinic thiokinase has

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been isolated in a highly purified form from *E. coli* by a procedure which is basically similar to those described by other laboratories (Gibson *et al.*, 1967; Ramaley *et al.*, 1967). Studies on the phosphorylation of the enzyme and isotopic exchange experiments lend further support to the sequence of reactions 1–3, with the added implication of an enzyme-bound CoA which may play a role in the formation of intermediary complexes. These studies are especially pertinent in view of the proposal of enzyme-bound CoA in a nonphosphorylated high-energy form of the enzyme (Upper, 1964; Cha *et al.*, 1965, 1967b; Moyer *et al.*, 1967).

Experimental Section

Materials. Sephadex G-150 (superfine) was purchased from Pharmacia Fine Chemicals and was prepared according to the manufacturer's recommendations. Whatman DE-23 (DEAE-cellulose) was purchased from Reeve Angel. ATP and ADP were obtained from Sigma. CoA (lithium salt) was a product of P-L Biochemicals. Succinyl-CoA was synthesized according to Simon and Shemin (1953) and purified and desalted by the procedure of Cha and Parks (1964). [2,3-^{14}C]Succinic acid and [^{32}P]P_i were purchased from New England Nuclear Corp. [8-^{14}C]ADP was a product of Schwarz BioResearch. [γ-^{32}P]ATP was synthesized enzymatically (Glynn and Chappell, 1964) and was also purchased from International Nuclear Corp. All labeled ATP and P_i used in this investigation was purified by DEAE-cellulose chromatography (Wehrli *et al.*, 1965).

Enzyme Assay. The assay system described by Kaufman *et al.* (1953) was used, except that dithiothreitol (1.5×10^{-4} M) was substituted for glutathione. Enzyme units and specific activity are expressed also as described.

Protein Determination. Protein was estimated by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. It was found that a purified enzyme solution shown to contain 1 mg/ml by this method was equivalent to 1.2 mg/ml according to the value of 0.511 found for A_{280} (1 mg/ml) at pH 7.2 by Ramaley *et al.* (1967).

Radioactivity Measurements. Paper electropherograms were counted in a Nuclear-Chicago Actigraph III scanner equipped with scaler. Other samples were mixed with a naphthalene–dioxane solution (Bray, 1960) and counted in a Nuclear-Chicago scintillation spectrometer.

Paper Electrophoresis. Whatman No. 3MM paper strips (2.54 × 75 cm) were moistened with appropriate buffer and placed on a Savant 30-in. flat-plate electrophoresis unit, cooled at 0°, where the samples were applied to the paper. For the separation of succinate from succinyl-CoA and of P_i, ATP, and the phosphorylated enzyme, the pH 7.4 buffer system of Cha *et al.* (1967b) was found most useful. Samples were subjected to 33 V/cm for 90 min. For the separation of ATP from ADP a buffer at pH 3.6 (Sato *et al.*, 1963) was employed at 40 V/cm for 90 min.

Dephosphorylation of Succinic Thiokinase. In order to prepare enzyme containing a minimal amount of covalently bound phosphoryl groups, succinic thiokinase was treated with ADP and succinate in the presence of Mg^{2+}. The reaction mixture contained enzyme (500 units), Tris-HCl (400 μmoles, pH 7.4), ADP (78 μmoles), $MgCl_2$ (110 μmoles), and disodium succinate (200 μmoles) in a final volume of 4.0 ml. After incubation for 30 min at 4° the solution was passed through a column of Sephadex G-50 (1.5 × 20 cm), previously equilibrated with 0.05 M Tris-HCl–0.1 M KCl–0.001 M EDTA (pH 7.4). The eluted enzyme solution was made 70% saturated with respect to ammonium sulfate at pH 7.8. After centrifugation the precipitated enzyme was redissolved in the eluting buffer.

Polyacrylamide Gel Electrophoresis of Enzyme Preparations. Polyacrylamide gel electrophoresis of various enzyme fractions was carried out at pH 8.5 essentially as described by Ornstein (1964). The gel columns were of an 0.7-cm diameter and a 2-ml volume. After electrophoresis at 15 mA/gel, the gels were stained with Amido Black. They were destained by shaking in 15% acetic acid in the presence of a small quantity of Dowex 1-Cl overnight. When ^{32}P-phosphorylated enzyme was analyzed, the gels were sliced after electrophoresis (Fairbanks *et al.*, 1965). One slice was stained with Amido Black and another subjected to radioautography (Kodak "Blue Brand" X-Ray film) overnight.

Results

Purification of Succinic Thiokinase. Succinic thiokinase was isolated from *E. coli* (ATCC 4517) by a procedure which employed steps described by Gibson *et al.* (1967) with some modification in the first stages. Subsequent to the ammonium sulfate fractionation, the enzyme was chromatographed on DEAE-cellulose, and following this, was subjected to gel filtration on Sephadex G-150. All operations described were carried out at 0–4°.

DEAE-cellulose chromatography was carried out using Whatman DE-23 ion exchanger. This material was washed according to the manufacturer's recommendations and equilibrated with 0.02 M potassium phosphate–0.02 M KCl–0.001 M EDTA buffer (pH 7.8). A column of the exchanger (3.5 × 40 cm) was poured and a flow rate of approximately 200 ml/hr was maintained. Succinic thiokinase from the ammonium sulfate fractionation was passed through a column of Sephadex G-25 to remove ammonium sulfate and then chromatographed. The controlled heat-denaturation step (Gibson *et al.*, 1967) was omitted. After application of the enzyme solution the column was washed with 400 ml of the equilibrating buffer. Elution was carried out with a linear chloride gradient established by placing 2.0 l. of 0.02 M potassium phosphate–0.05 M KCl–0.001 M EDTA buffer (pH 7.8) in the mixing vessel and 2.0 l. of 0.02 M potassium phosphate–0.275 M KCl–0.001 M EDTA buffer (pH 7.8) in the reservoir. The enzyme was eluted as a sharp peak in the center of the gradient. Peak fractions (those fractions which had a specific activity at least twofold higher than that of enzyme applied to the column) were pooled and precipitated by the addition of one volume of saturated ammonium sulfate (pH 7.8) and solid ammonium sulfate until a final concentration of 70% saturation was attained. The pH of the mixture was

maintained at 7.8 by the addition of concentrated NH_4OH. The enzyme was then collected by centrifugation at 25,000 rpm for 10 min in a Spinco Model L centrifuge using a 30 rotor. The precipitate was redissolved in a minimal volume of 0.05 M potassium phosphate–0.05 M KCl–0.001 M EDTA buffer (pH 7.8). One volume of saturated ammonium sulfate solution (pH 7.8) was added. Under these conditions the enzyme appeared to be stable indefinitely when kept at 0–4°.

Gel filtration of the DEAE-cellulose-fractionated enzyme on Sephadex G-150 was performed as the need for freshly purified succinic thiokinase arose. Sephadex G-150 (superfine) was swelled and equilibrated with 0.05 M potassium phosphate–0.05 M KCl–0.001 M EDTA buffer (pH 7.8), according to the manufacturer's recommendations. The gel was poured into a glass column (2.5-cm diameter) and a Buchler peristaltic pump was used to pack the gel in a downward direction at a flow rate of 12 ml/hr. The final column height was 60 cm. Enzyme samples of 4 ml (usually about 4000 units) were passed into the column in the upward direction and eluted with the buffer used to equilibrate the gel at a flow rate of 8 ml/hr. Succinic thiokinase appeared in the effluent at the beginning of the included volume. A second protein peak, more included than and separated from the thiokinase, contained nucleoside diphosphate kinase activity. Succinic thiokinase from the pooled peak fractions (usually 50% of the activity applied to the column) was precipitated and redissolved, as described for the DE-23 chromatographed enzyme, except that the enzyme was redissolved in 0.05 M Tris-HCl–0.1 M KCl–0.001 M EDTA buffer (pH 7.8). Just before use the enzyme was precipitated by several volumes of saturated ammonium sulfate (pH 7.8). The precipitate was carefully washed three times with about 10 ml of the same ammonium sulfate solution and, finally, redissolved in a minimal amount of the Tris buffer just described. The washing procedure in a phosphate-free environment was undertaken as a routine procedure.

The ammonium sulfate fractionated enzyme used as the starting material in the procedure described here had specific activities of 125–160 units/mg, and represented 40-55% of the succinic thiokinase activity in the crude extracts. The DEAE-cellulose-fractionated enzyme preparations had specific activities of 300–600 units/mg and approximately 25% of the activity applied to the column was recovered in the best fractions. Enzyme purified by Sephadex G-150 treatment had specific activities of 400–800 units/mg, representing about 50% of the activity passed through the column.

As will be noted in the procedure described, considerable quantities of ammonium sulfate are present in the purified enzyme fractions. The presence of this salt was needed to ensure maximal stability of the enzyme and has been found to have little, if any, effect on the expression of enzyme activity in this or related work. A consequence of the presence of ammonium sulfate is described in the accompanying paper (Grinnell and Nishimura, 1969).

Polyacrylamide Gel Electrophoresis of the Purified Enzyme. The relative homogeneity of different fractions from the enzyme purification procedure is shown in Figure 1. The three gels represented were taken from three different electrophoretic runs. When purified succinic thiokinase was phosphorylated by [^{32}P]ATP and the entire reaction mixture subjected to gel electrophoresis the result shown in Figure 2 was obtained. Note that a single stained band corresponding to a single radioactive band was obtained. This confirms the observations, made through different techniques by Cha *et al.* (1967b) and Ramaley *et al.* (1967), that the phosphorylated protein and succinic thiokinase are identical.

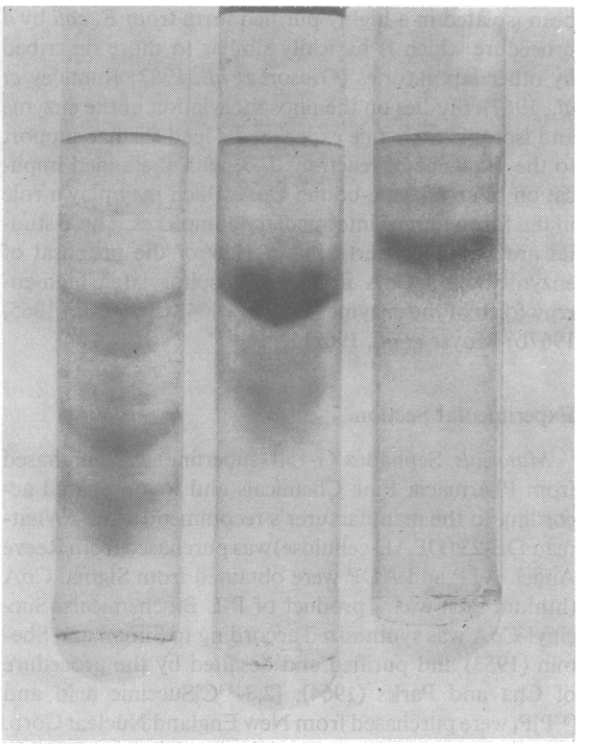

FIGURE 1: Polyacrylamide gel electrophoresis of various fractions from enzyme purification. Left: Ammonium sulfate fraction: 100 μg, specific activity 155; electrophoresis for 90 min. Center: DEAE-cellulose eluate: 180 μg, specific activity 580; electrophoresis for 45 min. Right: Sephadex G-150 fraction: 150 μg, specific activity 810; electrophoresis for 30 min. Other details are described in the Experimental Section. The fractions represented in this figure were not taken from the same batch.

Phosphorylation of the Enzyme by ATP and the Effect of CoA on Phosphorylation. It was of interest to examine the capacity of succinic thiokinase for phosphorylation. In Table I a series of experiments is summarized, showing that this capacity is the same whether precautions were taken to remove covalently bound P, or not. Also shown in the table are data which indicate that the presence of CoA at 2.5×10^{-5} M had no effect on the total amount of phosphorylation, but caused the release of significant quantities of P_i by activation of an apparent ATPase activity. The effect of CoA on phosphorylation of the enzyme was of interest in light of the report by Ramaley *et al.* (1967) that CoA stimulated the "slow" phosphorylation of *E. coli* succinic thiokinase at 0°. It will be noted that capacity for phosphorylation is related to enzyme activity and not to the amount of enzyme protein, although each of the enzyme preparations used

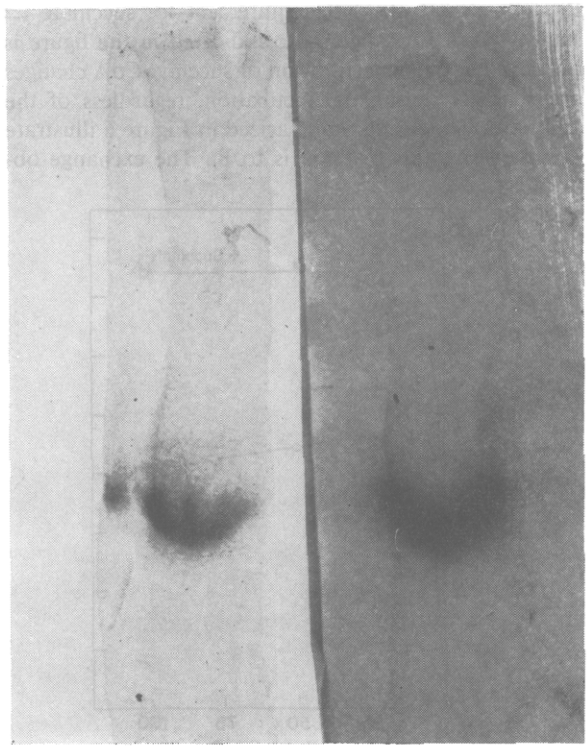

FIGURE 2: Polyacrylamide gel electrophoresis of ^{32}P-phosphorylated succinic thiokinase. The phosphorylation reaction mixture contained enzyme (20 units; specific activity 600), Tris-HCl (1.25 μmoles; pH 7.4), $MgCl_2$ (0.3 μmole), and [γ-^{32}P]ATP (15.6 mμmoles; 700,000 cpm), in a final volume of 0.053 ml. After incubation at 24° for 10 min, the entire solution was subjected to gel electrophoresis for 90 min. The gel was then sliced. Left: Gel slice stained with Amido Black. Right: Radioautogram of slice from the same gel. Other details are described in the Experimental Section.

was nearly homogeneous by the criterion of acrylamide gel electrophoresis.

Of paramount interest in Table I is that in the preparations of higher specific activity the phosphorylation of the enzyme approached 2 moles of P/mole of protein. The experiments described in Table I were preceded by a careful study of the effect of ATP concentration on phosphorylation of the enzyme, as carried out and reported by Ramaley *et al.* (1967). The concentration of ATP used in this investigation was close to saturating. In addition, it must be emphasized that a comparison of methodology used to determine phosphorylation by either ATP or P_i (in the presence of succinyl-CoA) gave almost identical results in the paper electrophoretic system or in the phenol extraction procedure (Ramaley *et al.*, 1967). In one series of experiments a purified enzyme preparation gave phosphorylations of 2.1 moles of P/mole of enzyme by ATP and 2.3 moles of P/mole of enzyme by P_i in the presence of succinyl-CoA. The latter result was obtained at an optimal succinyl-CoA concentration (Ramaley *et al.*, 1967).

The ATP ⇄ ADP Exchange Reaction. This reaction has been studied in the course of investigations of succinic thiokinase by several workers. Kaufman (1955) demonstrated an ATP ⇄ ADP interchange catalyzed by the spinach leaf enzyme. Aside from Mg^{2+}, no other components of the reaction were required. In fact, CoA was observed to inhibit the reaction. Subsequently, Upper (1964) showed that the exchange catalyzed by the *E. coli* enzyme was stimulated by CoA and, to a larger degree, by CoA and succinate, although succinate iself had little effect. Similar results were reported by Cha *et al.* (1967b) using the GTP ⇄ GDP system with pig heart succinic thiokinase. These investigators also observed significant stimulation by succinate alone.

The rate of phosphorylation of the enzyme by nucleo-

TABLE I: Phosphorylation of Succinic Thiokinase by ATP and Apparent ATPase Activity.[a]

Phosphorylation				ATPase			
Description of Enzyme Used		Moles of P Bound/Mole of enzyme protein[b]		Moles of P Bound/ 100 units of Enzyme		Moles of P Released/ Mole of P Bound to Enzyme	
Sp Act.	Pretreatment[c]	−CoA	+CoA	−CoA	+CoA	−CoA	+CoA
430	None	1.09 (0.91)	1.22 (1.02)	1.80	2.10	<0.1	4.8
500	Dephosphorylated	1.11 (0.92)	1.24 (1.03)	1.58	1.77	<0.1	4.3
780	None	1.99 (1.67)	1.85 (1.54)	1.76	1.64	<0.1	3.7
810	Dephosphorylated	2.23 (1.86)	1.96 (1.63)	1.96	1.81	<0.1	2.0
			Average	1.78	1.83		

[a] The reaction mixtures contained enzyme (5–16 units), Tris-HCl (2 μmoles, pH 7.4), $MgCl_2$ (0.2 μmole), [γ-^{32}P]-ATP (9.3 mμmoles; 1.54×10^5 cpm/mμmole), and, where added, CoA (1.0 mμmole with 2.0 mμmoles of dithiothreitol), in a final volume of 0.04 ml. After incubation for 10 min at 25°, the reaction was stopped by freezing. Aliquots of the solutions were analyzed for phosphorylated enzyme and P_i release by paper electrophoresis, as described in the Experimental Section. [b] The molecular weight of the enzyme was assumed to be 140,000; the values in parentheses were obtained on the basis of Δ_{280} (1 mg/ml) = 0.511 (Ramaley *et al.*, 1967). Other values were based on protein determination described in the Experimental Section. [c] Enzyme samples were dephosphorylated as described in the Experimental Section.

side triphosphate appears to be consistent with the participation of the phosphorylated enzyme as an intermediate in the reaction (Moyer *et al.*, 1967) Since the ATP ⇄ ADP exchange reaction is taken as indicating the reversible formation of phosphorylated succinic thiokinase, the ability of CoA and of CoA and succinate to stimulate exchange has been enigmatic. Our experiments show more dramatic stimulation of the ATP ⇄ ADP exchange reaction by CoA and by CoA and succinate than previously demonstrated.[1] This is illustrated in Figures 3 and 4.

Succinate ⇄ Succinyl-CoA Exchange Experiments. Kaufman (1955) first demonstrated an exchange of labeled succinate with succinyl-CoA in the presence of Mg^{2+} and a purified preparation of succinic thiokinase from spinach leaves. Although this reaction was enhanced by the addition of P_i, an absolute requirement for this anion was not demonstrated. In some elegant experiments Cha *et al.* (1967b) confirmed this result with the pig heart enzyme and simultaneously observed a stimulation of a CoA ⇄ succinyl-CoA exchange by P_i. On the other hand, Moyer *et al.* (1967) reported that P_i inhibited the succinate ⇄ succinyl-CoA exchange catalyzed by the *E. coli* enzyme. In view of these conflicting results and their implication in describing the intermediary sequences of the thiokinase reaction, we have also examined the exchange in detail. Figure 5 shows that

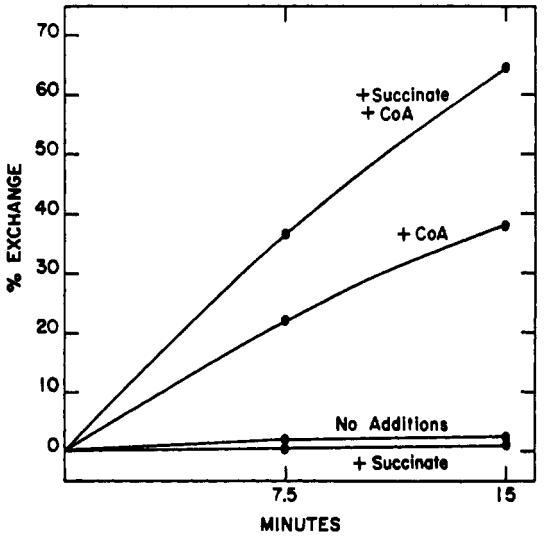

FIGURE 3: The effect of CoA and succinate on the ATP ⇆ ADP exchange reaction. The reaction mixtures contained enzyme (0.08 unit; specific activity 310), Tris-HCl (1.0 μmole, pH 7.4), $MgCl_2$ (0.1 μmole), ATP (0.1 μmole), [8-^{14}C]ADP (0.03 μmole, 0.04 μCi), and, where indicated, disodium succinate (0.1 μmole) and CoA (0.002 μmole, with 0.004 μmole of dithiothreitol), in a final volume of 0.020 ml. After incubation at 37° for the times indicated, the reaction vessels were chilled in ice water and 0.001 ml of cold 10% (w/v) trichloroacetic acid was added. Aliquots of 0.01 ml were then subjected to paper electrophoresis at pH 3.6 and analysis for radioactive ATP and ADP as described in the Experimental Section.

[1] The coenzyme A analog, desulfo-CoA, discussed in detail in the accompanying paper (Grinnell and Nishimura, 1969), stimulated the exchange slightly. This observation is of a preliminary nature and further studies are in progress.

P_i is an almost absolute requirement for succinate ⇄ succinyl-CoA exchange. Also indicated in the figure is the fact that the concentration of succinyl-CoA changes only slightly during the incubation, regardless of the additions. The results summarized in Figure 6 illustrate how sensitive this reaction is to P_i. The exchange ob-

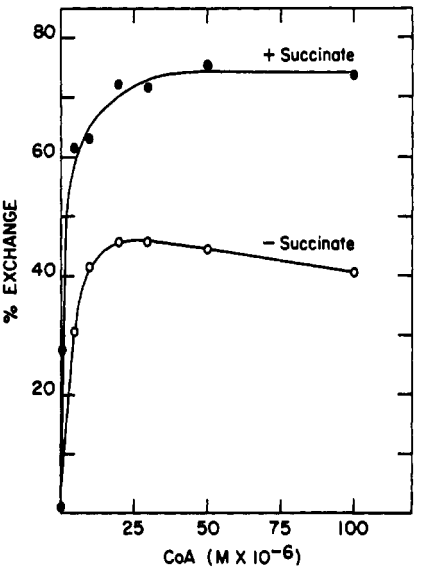

FIGURE 4: The effect of CoA concentration on the ATP ⇆ ADP exchange reaction in the presence and absence of succinate. The reaction components were the same as those described in Figure 3 except that dithiothreitol (2×10^{-4} M) was added throughout and CoA concentration was varied as indicated. The incubation time was 15 min and all other details were the same as those described in Figure 3.

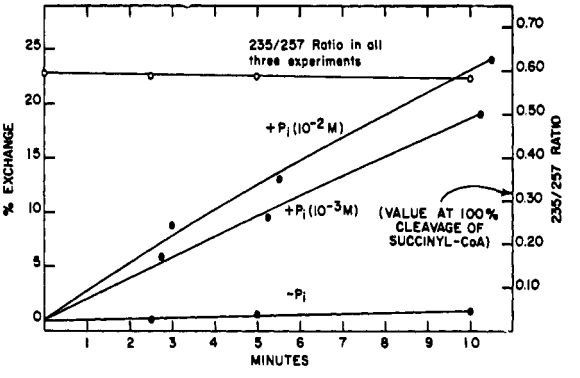

FIGURE 5: Stimulation of the succinate ⇆ succinyl-CoA exchange reaction by P_i. Quadruplicate sets of reaction mixtures contained enzyme (0.14 unit; specific activity 400), Tris-HCl (2.0 μmoles, pH 7.4), $MgCl_2$ (0.2 μmole), [2,3-^{14}C]succinate (0.290 μmole, 0.4 μCi), succinyl-CoA (0.049 μmole), and P_i, as indicated, in a final volume of 0.040 ml. Incubation was carried out at 25°. Aliquots of 0.010 ml were withdrawn from one set at the times indicated and analyzed for distribution of radioactivity in succinate and succinyl-CoA by paper electrophoresis at pH 7.4 and strip scanning, as described in the Experimental Section. The remaining three sets and a zero-time reaction mixture were used for the spectrophotometric determination of succinyl-CoA. The reactions were stopped at the indicated times by the addition of 0.96 ml of cold 0.01 N HCl. The solutions were then read in 1.0-ml cuvets (1-cm light path) against a blank containing all reaction components except succinyl-CoA in a Cary Model 11 spectrophotometer at 235 and 257 mμ.

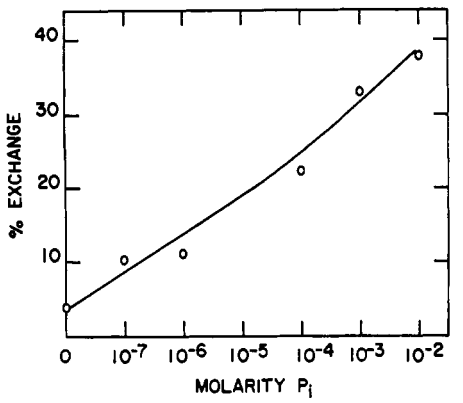

FIGURE 6: The effect of P_i concentration on the succinate ⇆ succinyl-CoA exchange reaction. The reaction mixtures contained enzyme (0.14 unit, specific activity 400), Tris-HCl (1.0 μmole, pH 7.4), $MgCl_2$ (0.1 μmole), [2,3-^{14}C]succinate (0.145 μmole, 0.2 μCi), succinyl-CoA (0.025 μmole), and P_i, as indicated, in a final volume of 0.020 ml. After incubation at 25° for 15 min the reaction vessels were chilled in ice water and aliquots of 0.010 ml were subjected to paper electrophoresis at pH 7.4 and radioactivity analysis as described in the Experimental Section.

served in the absence of added P_i is slightly variable from experiment to experiment, probably due to traces of P_i in the substrates and enzyme preparations. In any case, the stimulatory effect of P_i has been consistently reproducible.

Discussion

The results of phosphorylation by ATP or by P_i (in the presence of succinyl-CoA) of highly purified succinic thiokinase from *E. coli* indicate that close to 2 moles of phosphoryl groups are incorporated per mole of enzyme of highest specific activity. In purified enzyme preparations of lower specific activity phosphorylations closer to 1 mole of P/mole of protein is observed. Thus, in the numerous experiments conducted in this laboratory it has become evident that the extent of phosphorylation depends upon the activity of the particular enzyme preparation used. This is consistent with an earlier observation made in this laboratory (Nishimura, 1967) that the ability of phosphorylated succinic thiokinase to give up P, as either ATP or P_i, decreases as the enzyme loses catalytic activity. Ramaley *et al.* (1967) have reported that the extent to which the *E. coli* enzyme is phosphorylated depends upon the amount of protein present, regardless of the specific activity. Furthermore, these investigators found that phosphorylation of the enzyme by ATP yielded 1 mole of P/mole of protein and phosphorylation by P_i in the presence of succinyl-CoA gave 1.4–1.8 moles of P/mole of protein. It is of interest that the pig heart enzyme has an estimated molecular weight of approximately 70,000 (Cha *et al.*, 1967a), compared with the more accurately determined value of 141,000 for the *E. coli* enzyme (Ramaley *et al.*, 1967). However, it has also been estimated that the smaller mammalian enzyme has the capacity to bind at least two, and probably four, phosphoryl groups (Cha *et al.*, 1967b). The variations in phosphorylation of the *E. coli* enzyme described in this work and by Ramaley *et al.* (1967) may indicate that bacterial succinic thiokinase is highly susceptible to inactivation and that more sites for phosphorylation actually exist in the native enzyme.

The observation that phosphorylation by ATP was the same in the presence and absence of CoA at 2.5×10^{-5} M (see Table I) shows that under these conditions a high-energy nonphosphorylated enzyme (Cha *et al.*, 1965, 1967b; Moyer *et al.*, 1967) is probably not formed. It is difficult to understand why CoA by itself should stimulate ATP ⇄ ADP exchange. Perhaps enzyme-bound CoA acts to enhance turnover of the phosphoryl groups (dephosphorylation and rephosphorylation) of the protein by affecting the binding of either ATP or ADP, or both. The fact that CoA and succinate stimulated the ATP ⇄ ADP exchange to an even greater degree suggests that succinyl-CoA may also function in the same manner as CoA.[2] Since succinate itself did not stimulate the exchange and very little reaction occurred in the absence of CoA, it is suggested that the high endogenous ATP ⇄ ADP (or GTP ⇄ GDP) exchange observed in other laboratories (Kaufman, 1955; Cha *et al.*, 1967b) was due to the presence of small quantities of bound CoA in the enzyme preparations.

Thus, the results of this investigation indicate that noncovalently bound CoA (and possibly succinyl-CoA) may play an effector role in the phosphorylation of the enzyme and that this action of CoA may be distinguished from its reaction in the formation of a high-energy nonphosphorylated enzyme. The decisive stimulation by P_i of the succinate ⇄ succinyl-CoA exchange reaction (Figures 5 and 6) provides further evidence for reactions 2 and 3 and adds support to the proposal that enzyme-bound succinyl phosphate is an intermediate in the succinic thiokinase reaction.

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

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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.