Formation of Succinyl Phosphate by Reaction of Phosphorylated Succinic Thiokinase with Succinate*

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ABSTRACT: Previous work in this laboratory showed that succinyl phosphate can be isolated from reaction mixtures containing relatively large amounts of succinic thiokinase (isolated from *Escherichia coli*), adenosine triphosphate (ATP), succinate, and magnesium ions. In addition, it was demonstrated that chemically synthesized succinyl phosphate was utilized by the enzyme to form both ATP and succinyl-CoA. It was shown that the enzyme can also be phosphorylated by chemically synthesized succinyl phosphate. However, it was not clear whether the formation of enzyme-bound succinyl phosphate involved direct phosphorylation of succinate by ATP or whether the phosphorylated enzyme reacted with succinate. The present experiments demonstrate that the phosphorylated enzyme reacts with succinate to yield succinyl phosphate, which, in the absence of coenzyme A (CoA), dissociates from the enzyme. Incubation of the phosphorylated enzyme with both succinate and CoA leads to formation of inorganic phosphate accompanied by little, if any, formation of succinyl phosphate. The data support a reaction sequence in which the enzyme is phosphorylated by ATP and the phosphorylated enzyme reacts with succinate to yield enzyme-bound succinyl phosphate, which in turn reacts with CoA to give succinyl-CoA.

As there is also evidence favoring an alternative mechanism (the "high-energy" nonphosphorylated pathway), it may be suggested that both reaction sequences are catalyzed by the enzyme.

A recent report from this laboratory (Nishimura and Meister, 1965) presented evidence for the participation of succinyl phosphate as an intermediate in the reaction catalyzed by succinic thiokinase. Thus, succinyl

$$\text{succinyl-CoA}^{1} + \text{ADP} + P_i \rightleftharpoons \text{succinate} + \text{CoA} + \text{ATP}$$

phosphate was isolable when relatively large amounts of the enzyme were incubated with succinate, ATP, and magnesium ions. Furthermore, chemically synthesized succinyl phosphate was utilized by the enzyme to form ATP (in the presence of ADP) and succinyl-CoA (in the presence of CoA). In addition, significant phosphorylation of succinic thiokinase by chemically synthesized succinyl phosphate was observed. The phosphoryl enzyme obtained in this manner appeared to have properties in common with those exhibited by succinic thiokinase preparations phosphorylated by inorganic phosphate or by ATP (Bieber *et al.*, 1964; Kreil and Boyer, 1964). On the basis of these observations and the previously reported ^{18}O-exchange data (Cohn, 1951; Hager, 1957), the formation of enzyme-bound succinyl phosphate was suggested to follow either of two reaction mechanisms.

$$\text{enzyme} + \text{succinate} + \text{ATP} \rightleftharpoons \text{enzyme-succinyl phosphate} + \text{ADP} \quad (1)$$

or

$$\text{enzyme} + \text{ATP} \rightleftharpoons \text{enzyme-phosphate} + \text{ADP} \quad (2a)$$

$$\text{enzyme-phosphate} + \text{succinate} \rightleftharpoons \text{enzyme-succinyl phosphate} \quad (2b)$$

According to this representation, reaction of enzyme-succinyl phosphate with CoA would follow.

$$\text{enzyme-succinyl phosphate} + \text{CoA} \rightleftharpoons \text{enzyme} + \text{succinyl-CoA} + P_i \quad (3)$$

In the present investigation direct evidence for reaction 2b has been obtained. Phosphorylated enzyme was prepared by reaction of succinic thiokinase with either ATP or chemically synthesized succinyl phosphate. Incubation of the phosphorylated enzyme with succinate, in the absence of CoA, led to the synthesis of succinyl phosphate. When the phosphorylated enzyme was incubated with both succinate and CoA, P_i was formed and negligible quantities of succinyl phosphate were found. Addition of CoA at a time when succinyl phosphate formation was nearly complete resulted in no significant difference in the amount

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[1] Abbreviations used: ATP and ADP, adenosine 5′-tri- and -diphosphates, respectively; GTP and GDP, guanosine 5′-tri- and -diphosphates, respectively; CoA, coenzyme A; STK-P, phosphorylated succinic thiokinase.

of succinyl phosphate synthesized. It is concluded that, in the absence of CoA, succinyl phosphate dissociates from the enzyme. These results together with earlier data support a mechanism in which enzyme-bound succinyl phosphate is formed by reaction of succinate with a phosphorylated form of the enzyme.

Evidence has been presented by others for a pathway involving a high-energy nonphosphorylated form of the enzyme. This mechanism can be represented in the following way.

$$\text{ATP (GTP)} + \text{enzyme} \rightleftharpoons \text{phosphoryl enzyme} + \text{ADP (GDP)} \quad (4)$$

$$\text{phosphoryl enzyme} + \text{CoA} \rightleftharpoons \text{CoA-enzyme} + P_i \quad (5)$$

$$\text{CoA-enzyme} + \text{succinate} \rightleftharpoons \text{succinyl-CoA} + \text{enzyme} \quad (6)$$

This scheme was originally postulated by Upper (1964) on the basis of the fact that CoA stimulated ATP–P_i exchange and the removal of phosphorus from the phosphorylated enzyme. Cha *et al.* (1965) showed that most of the phosphorus could be removed from phosphorylated succinic thiokinase by incubation with CoA and that subsequent incubation of the dephosphorylated enzyme with succinate, followed by addition of NH_2OH, resulted in the formation of some succinyl hydroxamate. The published data indicate that the amount of succinyl hydroxamate formed in this manner was only about 20% of that which would have been expected on the basis of the phosphorylated enzyme present initially. Later, Cha *et al.* (1966) and Moyer and Smith (1966) reported experiments in which [^{3}H]CoA and [^{32}P]-dephospho-CoA were employed. Incubation of phosphorylated enzyme with either labeled compound led to release of phosphorus from the enzyme with the concomitant binding of the labeled compound to the enzyme. However, upon incubation of the labeled enzyme with succinate, relatively small quantities of label (as succinyl-CoA or succinyl dephospho-CoA) were released from the enzyme.

The apparent inconsistencies in the data, which have led to the postulation of two different pathways, may be explained by a mechanism involving alternative reactions catalyzed by the same enzyme. This possibility will be reviewed in the Discussion section.

Experimental Section

Materials. ADP, CoA, and *p*-hydroxymercuribenzoate (sodium salt) were purchased from the Sigma Chemical Co. Sephadex G-50 (medium) was obtained from Pharmacia, Piscataway, New Market, N. J., and prepared according to the manufacturer's recommendations. [γ-^{32}P]ATP was prepared enzymatically by reversal of the glutamine synthetase reaction (Wellner, 1963). [^{32}P]Phosphate was obtained from Atomic Energy of Canada, Ltd., Ottawa, Canada, and the New England Nuclear Corp. Chemically synthesized [^{32}P]succinyl phosphate was prepared as described previously (Nishimura and Meister, 1965). [^{3}H]ATP was a product of Schwarz BioResearch, Inc. [2,3-^{3}H]-Succinic acid was obtained from the New England Nuclear Corp. Glass vials for scintillation counting were products of the Wheaton Glass Co., Millville, N. J. Succinic thiokinase was isolated from *Escherichia coli* (ATCC 4517) according to the procedure described by Upper (1964). Specific activities of 500–850 μmoles of succinyl hydroxamate/mg of protein per 30 min were obtained. The assay method of Kaufman *et al.* (1953) was employed.

Preparation and Isolation of [^{32}P]Phosphorylated Succinic Thiokinase. The procedure for phosphorylation of the enzyme by ATP was essentially that employed by Upper (1964). The conditions of the incubation of [γ-^{32}P]ATP with succinic thiokinase are explained in the text. The reaction mixture (0.2–1.5 ml) was pipetted onto a column of Sephadex G-50 (2 × 23 cm) which had been equilibrated with Tris-HCl (0.05 M, pH 7.4). The column was eluted with the same buffer and fractions were collected at 4°. The labeled enzyme emerged in the 26–42-ml fraction, followed by ATP and P_i in the 65–105-ml fraction. The labeled enzyme was routinely precipitated by the addition of three volumes of saturated ammonium sulfate (saturated and adjusted to pH 7.2 with concentrated ammonium hydroxide at 30°). When stored in this form at 0° the phosphorylated enzyme was stable for at least 1 month. For each experiment an aliquot of the enzyme suspension was centrifuged at 20,000*g* for 20 min at 4°. The supernatant was carefully decanted and the precipitate containing the labeled enzyme was redissolved in a minimal quantity of 0.05 M Tris-HCl (pH 7.4).

Phosphorylation of succinic thiokinase by chemically synthesized succinyl phosphate was carried out in a volume of 1.0 ml containing $MgCl_2$ (5 μmoles) and Tris-HCl (100 μmoles) (pH 7.4), at 36° for 10 min. The reaction mixture was then subjected to gel filtration through Sephadex G-50, and the labeled enzyme was collected and stored, as described above for the ATP-phosphorylated enzyme. Further details are described under Results.

Analysis of Tritium and ^{32}P. Tris-HCl (1.0 ml of 0.05 M) (pH 7.4), containing the appropriately diluted sample, was mixed with 19 ml of Bray's (1960) solution in a glass counting vial. Radioactivity was determined in a Nuclear-Chicago liquid scintillation counter, calibrated for the simultaneous assay of ^{3}H and ^{32}P.

Electrophoretic Separation of Phosphorylated Enzyme from ATP, P_i, and Succinyl Phosphate. The method was in principle the same as that described by Cha *et al.* (1965, 1966, details unpublished). Aliquots of 10–30 μl were analyzed by paper electrophoresis in the system described previously (Nishimura and Meister, 1965). Electrophoresis was terminated when the picric acid marker had moved 20 cm toward the anode. The paper strips were then dried and counted in a Nuclear-Chicago Actigraph III strip counter equipped with scaler. In some experiments sections

TABLE I: Phosphorylation of Succinic Thiokinase by [γ-^{32}P]ATP.

Expt[a]	Reaction Vol. (ml)	[γ-^{32}P]ATP Incubd (mμmoles)	[^{32}P]STK-P Formed (mμmoles)	Enzyme Units in STK-P	Ratio of Enzyme Units:STK-P Formed
1	1.5	426	67	3580	52.5
2	1.5	843	118	3610	30.7
3	0.2	434[b]	14.5	545	37.6

[a] The reaction mixtures included enzyme, Tris-HCl (pH 7.4, 0.05 M), $MgCl_2$ (0.005 M), and [γ-^{32}P]ATP (4470–29,100 cpm/mμmole). After incubation at 4° for 30 min the samples were processed as described under Experimental Section. [b] Included 50 μc of [^{3}H]ATP. See text for explanation.

of the paper strips were eluted with water and counted by liquid scintillation counting to determine ^{3}H as well as ^{32}P. The phosphorylated enzyme remained close to the origin, while succinate, succinyl phosphate, P_i, and ATP migrated toward the anode.

Results

Phosphorylation of Succinic Thiokinase by ATP and by Chemically Synthesized Succinyl Phosphate. Succinic thiokinase was phosphorylated by incubation of the enzyme with [γ-^{32}P]ATP and magnesium ions. Separation of the enzyme from ATP and P_i was achieved by passage of the reaction mixture through a column of Sephadex G-50 (see Experimental Section). The results of three experiments are summarized in Table I. The concentrations of enzyme incubated were approximately the same, but the ATP concentrations were varied. At the lowest concentration of ATP (expt 1) the least phosphorylation was achieved. There was little difference in the yield of phosphorylated enzyme at the two higher concentrations of ATP (expt 2 and 3). Experiment 3 was carried out using [^{3}H,^{32}P]ATP. The amount of ^{3}H bound to the enzyme indicated that less than 0.03 mμmole of adenine, compared to 14.5 mμmoles of phosphorus, was associated with the enzyme.

Succinic thiokinase was also phosphorylated by chemically synthesized succinyl phosphate for the purpose of comparing the product with that obtained by reaction of ATP and enzyme. Experimental conditions are described in the Experimental Section. Of the 136 mμmoles (3.5×10^6 cpm) of succinyl phosphate incubated, 11 mμmoles of phosphorus was found in the enzyme fractions, which contained 1140 units of STK activity. This amounts to a ratio of enzyme units to STK-P formed of 104. Experiments using larger amounts of succinyl phosphate have not been attempted.

Reaction of ATP-Phosphorylated Enzyme with Various Substrates. The phosphorylated enzyme was tested for its ability to react with substrates of the thiokinase reaction. The results of this study are shown in Table II. Almost complete release of phosphorus from the enzyme ensued when the phosphoryl enzyme was allowed to react with ADP to form ATP, CoA or CoA and succinate to form P_i, and succinate to form P_i and succinyl phosphate. Incubation of the phosphoryl enzyme with Mg^{2+} alone did not result in the release of significant phosphorus. Malonate, which

TABLE II: Reaction of ATP-Phosphorylated Succinic Thiokinase with ADP, CoA, and Succinate.

Reaction Mixtures[a]	Products Formed (mμmoles) ATP	P_i	Succinyl Phosphate
STK-P alone	0.001	0.004	<0.001
STK-P alone[b]	0.006	0.002	<0.001
STK-P + Mg^{2+}	0.011	<0.001	<0.001
STK-P + Mg^{2+} + ADP	0.145	0.004	<0.001
STK-P + Mg^{2+} + CoA	<0.001	0.133	<0.001
STK-P + Mg^{2+} + succinate	<0.001	0.120	0.024
STK-P + Mg^{2+} + CoA + succinate	0.004	0.142	<0.001
STK-P + Mg^{2+} + malonate	0.001	0.002	<0.001

[a] The reaction mixtures contained enzyme (10.0 units, 0.151 mμmole of bound phosphorus, 6250 cpm), Tris-HCl (pH 7.4, 10 μmoles), $MgCl_2$ (0.5 μmole), ADP (0.1 μmole), CoA (0.01 μmole), disodium malonate (10 μmoles), and disodium succinate (10 μmoles) as indicated in the table in a final volume of 0.1 ml, pH 7.4; after incubation for 10 min a 36°, the reaction mixtures were chilled to 0°. Aliquots of the solutions were subjected to paper electrophoresis and analyzed as described under Experimental Section. [b] The enzyme was not incubated.

TABLE III: Reaction of ATP-Phosphorylated Succinic Thiokinase with Succinate to Form Inorganic Phosphate and Succinyl Phosphate.[a]

Expt	Succinate Added (μmoles)	CoA Added (μmole)	Enzyme Added			Time (sec)	Products Formed (mμmole)	
			Units	Bound P mμmole	Bound P cpm		P_i	Succinyl Phosphate
						0[b]	0.01	<0.01
1	0.03	0	11.3	0.366	1650	60	0.02	0.02
						120	0.03	0.02
2	0.30	0	11.3	0.366	1650	60	0.06	0.04
						120	0.10	0.09
						30	0.13	0.12
3a	3.00	0	11.3	0.366	1650	60	0.15	0.15
						120	0.16	0.17
3b	3.00	0	30.0	0.980	4650	60[c]	0.11	0.12
						60[d]	0.03	0.02
3c		0	4.3	0.139	500	180	0.06	0.07
	3.00	0.004				180[e]	0.06	0.07
		0.004				120	0.12	<0.001
4[f]	0	0	19.0	0.221	3520	0	<0.001	<0.001
	0	0				120	<0.001	<0.001
	2.00	0				120	0.059	0.143
	2.00	0.002				120	0.210	<0.001

[a] The reaction mixtures contained ATP-phosphorylated enzyme, Tris-HCl (pH 7.4, 3 μmoles), $MgCl_2$ (0.15 μmole), and disodium succinate, as indicated, in a final volume of 0.029 ml, pH 7.4, at 36°. After incubation for the specified times, the reaction was stopped by addition of 0.005 ml of a *p*-hydroxymercuribenzoate suspension (sodium salt, 2.2 mg/ml). Aliquots of the solutions were analyzed as described under Experimental Section. [b] The enzyme was treated with *p*-hydroxymercuribenzoate before addition to the reaction mixture. [c] The incubation temperature was 23°. [d] The incubation temperature was 0°. [e] CoA was added at 120 sec. [f] The incubation volume was 0.020 ml. Succinyl phosphate phosphorylated enzyme was employed.

acts as a competitive inhibitor of succinate,[2] had no effect on the phosphoryl enzyme. The data are for the most part in agreement with those obtained with the pig heart enzyme by Cha *et al.* (1965). These workers found that phosphorus was released from the enzyme by GDP to form GTP, by CoA to form P_i, and by succinate and CoA to yield succinyl-CoA and P_i. Inorganic phosphate was also released by succinate alone. In the latter experiment some succinyl hydroxamate was formed upon the addition of hydroxylamine, but no correlation was made between this result and succinyl phosphate formation.

An interesting observation was made regarding the enzymatic activity of the phosphorylated enzyme. Phosphorylated enzyme as obtained from the Sephadex G-50 column (see Experimental Section) rapidly lost enzymatic activity after 1–2 days. The loss of enzymatic activity was accompanied by a proportional decrease in the total amount of phosphorus which was releasable by incubation with ADP, CoA, or succinate. The phosphorylated enzyme could be stabilized by addition of saturated ammonium sulfate, as described under Experimental Section.

Reaction of ATP-Phosphorylated and Succinyl Phosphate Phosphorylated Enzyme with Succinate. As the amount of succinyl phosphate found in the experiment described above was small in relation to the quantity of P_i formed, conditions for optimal synthesis of succinyl phosphate were sought. Experiments designed to this end using both ATP-phosphorylated enzyme and succinyl phosphate phosphorylated enzyme are described in Table III. *p*-Hydroxymercuribenzoate was used to stop the reaction. Addition of this inhibitor did not result in the loss of phosphorus from the enzyme and at the concentration of mercurial used it was instantaneously effective. Experiments 1–3 summarize the reactions of ATP-phosphorylated enzyme and expt 4 describes reactions of the succinyl phosphate phosphorylated enzyme. At succinate concentrations of 1.04×10^{-3} and $\times 10^{-2}$ M (expt 1 and 2, respectively) formation of inorganic phosphate and succinyl phosphate in approximately equal amounts was observed. Rapid formation of inorganic phosphate and succinyl phosphate was observed at a succinate concentration of 0.104 M (expt 3a). The effect of lower incubation

[2] J. S. Nishimura, unpublished observation.

temperatures at the same succinate concentration is shown in expt 3b. A relatively slow reaction was observed at 23° and a very sluggish one at 0°. In some experiments there were indications that succinyl phosphate, once formed, was not tightly bound to the enzyme. One such experiment is described in expt 3c. Of interest in this case is that the addition of CoA at 2 min (when succinyl phosphate formation was essentially complete) had no effect on the results. This indicates that under the conditions of this experiment succinyl phosphate was released from the enzyme. Rebinding of succinyl phosphate to the enzyme might then be inhibited by the large amount of succinate present; reaction with CoA would be thereby prevented. In expt 4 reaction of succinyl phosphate phosphorylated enzyme with succinate resulted in a significant difference in the amounts of succinyl phosphate and P_i formed. The yield of succinyl phosphate was 71% of the total phosphorus released.

The appearance of inorganic phosphate accompanying succinyl phosphate formation can probably be ascribed to nonenzymatic hydrolysis of the latter during the incubation. Table IV describes an experiment in which the fate of chemically synthesized succinyl phosphate was studied under conditions designed to simulate those employed in the experiments with the phosphorylated enzyme. As shown in the table, approximately one-third of the succinyl phosphate added was hydrolyzed in 2 min at 36°, whether the enzyme was present or not. The amount of succinyl phosphate hydrolyzed agrees on a percentage basis with the amount of inorganic phosphate formed during reaction of the succinyl phosphate phosphorylated enzyme with succinate (see Table III, expt 4). Nonenzymatic hydrolysis alone does not explain the relatively high amounts of P_i liberated when ATP-phosphorylated succinic thiokinase was incubated with succinate (see Table III, expt 1–3). An explanation for this discrepancy could lie in the possibility that the enzyme contains bound CoA, which is not free to react with covalently bound phosphorus in the absence of succinate. However, upon addition of succinate the succinyl phosphate which is formed may react with the bound CoA, forming succinyl CoA and P_i. Accordingly, succinyl phosphate phosphorylated enzyme would be expected to contain less bound CoA.

Experiments with phosphorylated enzyme and [^{3}H]succinate have revealed no evidence for significant binding of succinate to the enzyme. In these experiments the use of large quantities of labeled succinate was required in order to effect release of phosphorus from the enzyme. Detection of [^{3}H]succinyl hydroxamate, [^{3}H]succinyl-CoA, or [^{3}H]succinyl phosphate was not possible, due to "tailing" of succinate into the succinyl phosphate area on electropherograms and the presence of radiochemical impurities in the succinyl-CoA and succinyl hydroxamate areas. Attempts to purify labeled succinate by Dowex 1 chromatography resulted in purer samples of the dicarboxylic acid, but subsequent analyses were plagued by what appeared to be spontaneously formed radiochemical impurities. The availability of tritiated succinic acid of much higher specific activity (138 μc/μmole) in these experiments than ^{14}C-labeled succinic acid (9–15 μc/μmole) dictated the use of the tritiated compound in this investigation. However, experiments employing [^{14}C]succinate and larger amounts of phosphorylated enzyme are contemplated.[3]

TABLE IV: Stability of Chemically Synthesized Succinyl Phosphate.

Reaction Mixtures[a]	Succinyl Phosphate Found after Incubn (%)
Succinyl phosphate + Mg^{2+}	100[b]
Succinyl phosphate + Mg^{2+}	70
Succinyl phosphate + Mg^{2+} + enzyme	73
Succinyl phosphate + Mg^{2+} + succinate + enzyme	67

[a] The reaction mixtures contained chemically synthesized [^{32}P]succinyl phosphate (1.9 mμmoles, 129,000 cpm), Tris-HCl (pH 7.4, 2.5 μmoles), $MgCl_2$ (0.1 μmole), disodium succinate (2 μmoles), and enzyme (5.5 units) as indicated in the table in a final volume of 0.02 ml, pH 7.4; after incubation for 2 min at 36°, 0.005 ml of a *p*-hydroxymercuribenzoate suspension (sodium salt; 2.2 mg/ml) was added. The reaction mixtures were chilled to 0°. Aliquots of 0.001 ml were subjected to paper electrophoresis and analyzed as described under Experimental Section. [b] The reaction mixture was not incubated.

Discussion

The data presented earlier (Nishimura and Meister, 1965) and here indicate that synthesis of succinyl-CoA from ATP, CoA, and succinate by succinic thiokinase proceeds *via* reactions 2a, 2b, and 3. The present demonstration that succinyl phosphate is formed by reaction of succinate with the phosphorylated enzyme and the fact that there is no ADP in the phosphorylated form of the enzyme render unlikely direct phosphorylation of succinate by ATP. The rates of succinyl phosphate formation from phosphorylated enzyme and succinate appear to be relatively slow compared to the reaction of phosphorylated enzyme with CoA and

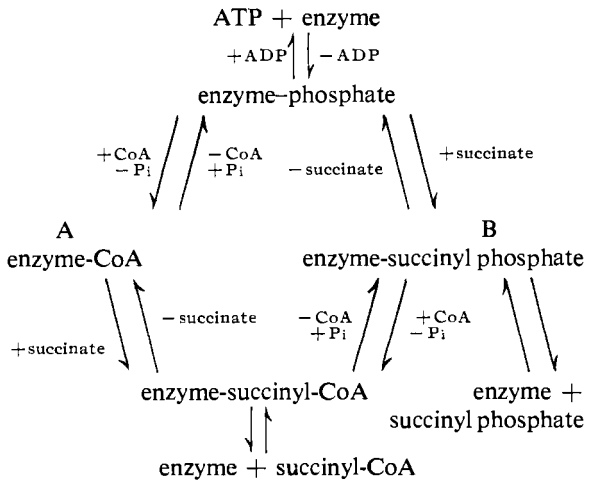
[3] In experiments in which succinyl phosphate phosphorylated enzyme was incubated with relatively low concentrations of [^{14}C]succinate (0.001 M) in the presence of CoA, succinyl-CoA and P_i were formed in approximately stoichiometric amounts. In one such experiment the cleavage of 0.22 mμmole of phosphorus from STK-P was accompanied by the synthesis of 0.20 mμmole of succinyl-CoA. As expected from the data presented in Table III, [^{14}C]succinyl phosphate (determined as succinyl hydroxamate) synthesis under the same conditions, but in the absence of CoA, was slight (<0.02 mμmole).

succinate. It must be emphasized, however, that the reaction mechanism, as written, is a simplification and is put forth to indicate a sequence of reaction steps and not necessarily an order in which substrates become bound to the enzyme. It is possible that CoA facilitates the binding of succinate to the enzyme; if this is true, the rate of reaction of succinate in the absence of CoA with the phosphorylated enzyme might be expected to be slower than that of the over-all reaction.

The rapid release of phosphorus from the phosphorylated enzyme by CoA and the concomitant binding of CoA to the enzyme cannot be explained in terms of the succinyl phosphate mechanism but is consistent with the mechanism represented by reactions 4–6. It is conceivable that the enzyme catalyzes two alternative reaction sequences, *i.e.*, both the succinyl phosphate and the high-energy nonphosphorylated pathways. The over-all reaction is depicted in Scheme I. The high-energy nonphosphorylated and succinyl phosphate pathways are labeled A and B, respectively. Both would have in common the phosphorylated form of the enzyme (STK-P) in which the phosphorylated site is 3-phosphohistidine in peptide linkage with the protein (Hultquist *et al.*, 1966). Both alternatives could be consistent with the ^{18}O-exchange data. This is obvious in the case of pathway B, and analogous explanations (Graves and Boyer, 1962; Falcone and Boyer, 1959) can be invoked for pathway A.

It would appear that an evaluation of the relative importance of sequences A and B in the over-all reaction could be made by an intensive study of the ^{18}O-transfer reaction. An experiment in which ^{18}O from inorganic phosphate was traced into CoA has been alluded to in a recent report (Boyer *et al.*, 1966); however, the details of this study and evidence concerning the manner in which ^{18}O can be subsequently transferred to succinate are not yet at hand. According to sequence A, reaction of phosphorylated enzyme with succinate and CoA should not result in the direct transfer of an oxygen atom from succinate to inorganic phosphate but, rather, to either CoA or a site on the protein. Then subsequent rephosphorylation of the enzyme and reaction of the latter with CoA and succinate would result in transfer of the oxygen atom from CoA or the enzyme to inorganic phosphate. In contrast, reaction according to sequence B would be expected to yield a direct transfer of oxygen from succinate to P_i. It may be possible to exploit these differences in experiments in which phosphorylated enzyme and ^{18}O-labeled succinate are employed and the kinetics of ^{18}O transfer are followed. Although such experiments would involve the use of considerable amounts of phosphorylated enzyme (much larger than those used in most experiments described to date) and difficult methodology, they may provide long-awaited answers to questions which might, otherwise remain unresolved.

SCHEME I



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