Evidence for Succinyl Phosphate as an Enzyme-bound Intermediate in the Reaction Catalyzed by Succinyl Coenzyme A Synthetase*

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ABSTRACT: When purified succinyl coenzyme A synthetase from *Escherichia coli* was incubated with [^{14}C]succinyl phosphate and coenzyme A, [^{14}C]succinyl coenzyme A was formed. Incubation of the enzyme with [^{32}P]succinyl phosphate and adenosine 5′-diphosphate led to adenosine 5′-triphosphate (ATP) synthesis. When the enzyme was incubated with [γ-^{32}P]ATP and succinate evidence was obtained for synthesis of succinyl phosphate, which was separated and identified by paper electrophoresis. Evidence that the enzyme is phosphorylated by succinyl phosphate was also obtained. The data support the conclusion that enzyme-bound succinyl phosphate is an intermediate in the reaction.

Several recent investigations have dealt with the mechanism of the reaction catalyzed by succinyl coenzyme A synthetase:

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

Hager (1962), in an excellent review of this subject, has summarized the available evidence for and against a mechanism involving (1) enzyme-bound succinyl phosphate, (2) enzyme-bound phosphoryl coenzyme A, and (3) no discrete intermediates.

The present studies were stimulated by earlier work in this laboratory on tripeptide synthetase in which direct evidence for the intermediate formation of an enzyme-bound dipeptide–phosphate anhydride was achieved (Nishimura *et al.*, 1963, 1964), and on glutamine synthetase in which data giving strong support for an enzyme-bound glutamyl phosphate intermediate were obtained (Krishnaswamy *et al.*, 1962; Khedouri *et al.*, 1964). In both of these reactions (Boyer *et al.*, 1956; Kowalsky *et al.*, 1956; Strumeyer, 1959) and in the reaction catalyzed by succinyl coenzyme A synthetase (Hager, 1962) it has been shown that ^{18}O is transferred from inorganic phosphate to a carboxyl group or vice versa. Although the ^{18}O studies can be interpreted in more than one way, they are clearly consistent with intermediate formation of an acyl phosphate.

In the experiments reported here, the enzyme was incubated with succinyl phosphate labeled with ^{14}C and ^{32}P; evidence was obtained for utilization of succinyl phosphate both for the synthesis of ATP[1] and for the formation of succinyl coenzyme A. Furthermore, when the enzyme was incubated with [γ-^{32}P]ATP and unlabeled succinate in the absence of added coenzyme A, evidence was obtained for the formation of succinyl phosphate. It is clear that further experiments are needed on the phenomena reported here; however, we believe that the findings are of significance in relation to the mechanism of this reaction and that their publication may be of value to those interested in this problem.

Although our data indicate that succinyl phosphate can be formed and can be utilized by the enzyme, the participation of other enzyme-bound intermediates cannot be excluded. A recent report (Kreil and Boyer, 1964) indicates that succinyl coenzyme A synthetase can be phosphorylated by inorganic phosphate in the presence of succinyl coenzyme A and also by ATP. The relationship of these observations to the present data is not yet entirely clear; however, as reported here, incubation of succinyl phosphate with the enzyme also leads to substantial phosphorylation of the enzyme. The significance of these observations is discussed here.

Experimental

Materials. [2,3-^{14}C]Succinic acid was obtained from the New England Nuclear Corp. Coenzyme A, ATP, and ADP were obtained from Sigma Chemical Co. [^{32}P]Phosphate was obtained from Atomic Energy of Canada, Ltd., Ottawa, Canada. *N,N′*-Dicyclohexylcarbodiimide was obtained from Schwarz BioResearch, Inc. [γ-^{32}P]ATP was prepared enzymatically with glutamine synthetase (Wellner, 1963).

Succinyl CoA synthetase was isolated from

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[1] Abbreviations used in this work: ATP and ADP, adenosine 5′-tri- and -diphosphates, respectively; CoA, coenzyme A; ATPase, adenosine triphosphatase.

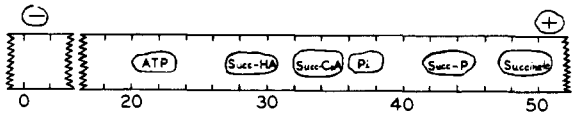

FIGURE 1: Paper-electrophoretic separation of succinyl phosphate and other compounds at *p*H 7 (see text for details). Key: Succ-HA, succinyl monohydroxamic acid; Succ-CoA, succinyl coenzyme A; P_i, inorganic phosphate; Succ-P, succinyl phosphate; 0 = origin; distances from origin are given in cm.

Escherichia coli (ATCC 4517) grown aerobically on a medium (Knight, 1961) containing succinate as the major source of carbon.[2] The enzyme was purified as described by Upper (1964), whose procedure was carried through the ammonium sulfate–fractionation step. The enzyme exhibited a specific activity in the range of 200–500 μmoles of succinyl hydroxamate per mg of protein per 30 minutes; activity was determined as described by Kaufman *et al.* (1953).

Synthesis of [^{14}C]*Succinyl Phosphate and* [^{32}P]*-Succinyl Phosphate.* [2,3-^{14}C]Succinic acid (26 mg; specific activity 9.1 mc/mmole) was converted to succinic anhydride essentially as described (Fieser and Martin, 1943). The labeled succinic acid was refluxed with 1 ml of freshly distilled acetyl chloride for 14 hours; after removal of the acetyl chloride by lyophilization, 1 ml of benzyl alcohol was added and the solution was heated under anhydrous conditions for 7 hours at 104°. The monobenzyl ester of [^{14}C]succinic acid was removed by extraction with saturated sodium bicarbonate. Approximately half the radioactivity remained in the organic phase and virtually all of this material was found to be the dibenzyl ester of succinic acid; this was subsequently recovered, after treatment with hydrogen and palladium catalyst, as succinic acid. The bicarbonate extract was acidified by addition of HCl and the monobenzyl ester of succinic acid was extracted with ether; the ether was evaporated and the ester was stored at −10°.

Benzyl succinyl phosphate was prepared from [^{14}C]-succinic acid monobenzyl ester and also from unlabeled succinic acid monobenzyl ester by the general procedure previously employed for the preparation of γ-glutamyl-α-aminobutyryl phosphate (Nishimura *et al.*, 1964) and β-aminoglutaryl phosphate (Khedouri *et al.*, 1964). A mixture of succinic acid monobenzyl ester (24 μmoles) and phosphoric acid (25 μmoles) in 0.12 ml of 75% aqueous pyridine was shaken with 0.2 ml of a solution of *N,N'*-dicyclohexylcarbodiimide (prepared by dissolving 0.5 g of the diimide in 0.5 ml of pyridine) at 0° for 2 hours. The entire reaction mixture was then streaked along a 13-cm line on a sheet of Whatman 3MM paper (25 × 76 cm) which had been previously moistened with a solution of 0.06 M acetic acid adjusted to *p*H 4.3 by addition of concentrated NH_4OH. Paper electrophoresis was carried out in a Savant "30-inch" flat plate unit at 0° and 40 v/cm. A marker consisting of picric acid was used, and when the marker had moved 17–18 cm from the origin toward the positively charged electrode (approximately 2 hours) the procedure was discontinued. The paper sheet was rapidly scanned while still damp in order to locate the radioactivity. In some preparations, radioautography was carried out. Under these conditions benzyl succinyl phosphate was located in a band which had moved 14–20 cm toward the positively charged electrode; it was completely separated from inorganic phosphate and from succinic acid monobenzyl ester, which moved, respectively, 22–27 and 6–11 cm toward the positively charged electrode. The portion of the paper containing benzyl succinyl phosphate was cut out and eluted with water at 4°, and the eluate was concentrated by flash evaporation. The product was stored at −15°. The overall yield of labeled benzyl succinyl phosphate was 30–40% based on the amount of radioactive material used.

Conversion of benzyl succinyl phosphate to succinyl phosphate was accomplished by catalytic hydrogenation. A solution (0.5 ml) of benzyl succinyl phosphate was mixed with 0.2 ml of 1 M Tris-HCl buffer (*p*H 7.4). Approximately 0.2 g of palladium catalyst was added and hydrogenation was carried out at 0° for 10 minutes. The solution was used immediately after removal of the catalyst by centrifugation.

Succinyl phosphate was also prepared from unlabeled starting materials by the procedure described by Kaufman (1954); this was used as carrier in some of the experiments described here.

Separation of Succinate and Succinyl Coenzyme A. The samples were subjected to paper electrophoresis at *p*H 3.6 essentially as described by Sato *et al.* (1963) except that zinc ion was not added to the buffer. The buffer consisted of 0.035 M citric acid and 0.0148 M sodium citrate. Electrophoresis was carried out on Whatman 3MM paper (2.54 × 76 cm) at 40 v/cm at 0° for 105 minutes. Under these conditions succinate moved 7.5–11.5 cm and succinyl coenzyme A moved 15–18 cm toward the positively charged electrode.

Separation of Succinyl Phosphate, Inorganic Phosphate, and ATP. The samples were subjected to paper electrophoresis using a buffer consisting of 0.05 M Tris, 0.05 M maleic acid, and 0.001 M EDTA adjusted to *p*H 7 by addition of sodium hydroxide. Electrophoresis was carried out on Whatman 3MM paper (2.54 × 76 cm) at 40 v/cm at 0° for 120–150 minutes. Under these conditions ATP, inorganic phosphate, and succinyl phosphate were separated (Figure 1). These compounds were located on the paper strip by application of the spray reagent described by Hanes and Isherwood (1949).

Determination of ATP. The samples (deproteinized, acidified; see Table II) were treated with 12 mg of acid-washed Norit A, and after brief shaking the mixtures were centrifuged. The sedimented charcoal was washed with water, and the ATP was eluted from the charcoal

[2] The cells were grown in 580-liter batches at the New England Enzyme Center, Tufts University School of Medicine.

with three portions of 2 ml each of 50% ethanol containing 0.3 M ammonium hydroxide. After addition of carrier ATP and ADP, the combined eluates were concentrated to dryness under reduced pressure; the residues were dissolved in water and subjected to descending paper chromatography on strips of Whatman 3MM paper as described by Krishnaswamy *et al.* (1962) in a solvent consisting of 1% ammonium sulfate–2-propanol (1 : 2).

Results

Utilization of Succinyl Phosphate for Synthesis of Succinyl Coenzyme A and ATP. When the enzyme was incubated with [^{14}C]succinyl phosphate and coenzyme A, significant quantities of succinyl coenzyme A were formed (Table I). Under these conditions much lower or negligible amounts of succinyl coenzyme A were formed when the enzyme was omitted, when coenzyme A was omitted, or when the succinyl phosphate preparation was hydrolyzed prior to the experiment by heating at 100° for 2 minutes. Succinyl coenzyme A formation was not affected by addition of 0.01 μmole of ADP, while addition of 1.0 μmole of ADP decreased formation of succinyl coenzyme A by about 50%. As indicated in Table I, the formation of succinyl coenzyme A was dependent, under these conditions, upon the amount of enzyme added. In addition to characterization of succinyl coenzyme A by paper electrophoresis, the product considered to be succinyl coenzyme A was further characterized by treatment with hydroxylamine. After treatment of the areas of the paper strips containing the product with 8 M hydroxylamine followed by elution with water, electrophoresis indicated that virtually all of the radioactivity moved as succinyl hydroxamate.

Data indicating utilization of succinyl phosphate for ATP synthesis are given in Table II. In these experiments the enzyme was incubated with [^{32}P]succinyl phosphate and ADP. Significant amounts of ATP were formed under these conditions but not in control experiments in which hydrolyzed succinyl phosphate was

TABLE I: Utilization of [^{14}C]Succinyl Phosphate for Synthesis of Succinyl Coenzyme A.

Reaction Mixtures[a]	Succinyl CoA Formed (mμmoles)
Enzyme + succinyl phosphate + CoA	11.3
Enzyme + succinyl phosphate + CoA + ADP (0.01 μmole)	11.7
Enzyme + succinyl phosphate + CoA + ADP (1.0 μmole)	5.9
Enzyme + succinyl phosphate	0.6
Succinyl phosphate + CoA	<0.2
Enzyme + succinyl phosphate (hydrolyzed)[b] + CoA	0.2
Enzyme[c] + succinyl phosphate + CoA	2.1
Enzyme[d] + succinyl phosphate + CoA	1.5

[a] The reaction mixtures contained enzyme (450 units), Tris-HCl (*p*H 7.4; 20 μmoles), $MgCl_2$ (1 μmole), 2-mercaptoethanol (2 μmoles), coenzyme A (0.26 μmole), ADP, and [^{14}C]succinyl phosphate (51 mμmoles; 230,000 cpm) as indicated in the table in a final volume of 0.2 ml, *p*H 7.4; after incubation for 5 minutes at 17°, 0.02 ml of 1.8 M $HClO_4$ was added and the precipitated protein was removed by centrifugation. Aliquots of the supernatant solutions were subjected to paper electrophoresis at *p*H 3.6 as described under Experimental. [b] Hydrolyzed prior to the experiment by heating at 100° for 2 minutes. [c] Enzyme, 134 units. [d] Enzyme, 67 units.

TABLE II: Formation of ATP from Succinyl Phosphate and ADP.

Reaction Mixtures[a]	ATP Formed (mμmoles)
Enzyme + succinyl phosphate + ADP	0.23
Enzyme + succinyl phosphate (hydrolyzed)[b] + ADP	<0.005
Enzyme + succinyl phosphate[c] + ADP	0.40
Enzyme + succinyl phosphate (hydrolyzed)[b,c] + ADP	<0.005
Enzyme[d] + succinyl phosphate + ADP	0.13
Enzyme[d] + succinyl phosphate (hydrolyzed)[b] + ADP	<0.005

[a] The reaction mixtures contained enzyme (174 units), [^{32}P]succinyl phosphate (20 mμmoles; 49,000 cpm), Tris-HCl (15 μmoles), $MgCl_2$ (1 μmole), 2-mercaptoethanol (2 μmoles), and ADP (0.1 μmole) as indicated in the table in a final volume of 0.2 ml, *p*H 7.4. After incubation at 17° for 5 minutes, 1.0 ml of 0.125 M trichloroacetic acid was added and the precipitated protein was removed by centrifugation. Aliquots of the supernatant solutions were made 1 M with respect to NH_2OH (at *p*H 7) and allowed to stand at 25° for 15 minutes. The solutions were then made acid by addition of trichloroacetic acid and processed further for the determination of ATP as described under Experimental. No ATP was found in control experiments in which enzyme or ADP was omitted. [b] Hydrolyzed prior to the experiment by heating at 100° for 2 minutes. [c] Succinyl phosphate, 60 mμmoles. [d] Enzyme, 87 units.

used, or in experiments in which the enzyme was omitted. Paper chromatographic study of the ATP isolated from the reaction mixtures by adsorption on charcoal indicated that more than 90% of the adsorbed radioactivity was present as ATP and therefore that relatively little radioactivity was incorporated into ADP.

Evidence for the Enzymatic Synthesis of Succinyl Phosphate. Relatively large amounts of enzyme were incubated with [γ-^{32}P]ATP and succinate for 45 seconds (Table III). The solutions were deproteinized and then subjected to paper electrophoresis as described under Experimental. The radioactivity in the area corresponding to succinyl phosphate (located by means of the Hanes-Isherwood spray) was determined. Control experiments were carried out in which hydroxylamine was added prior to electrophoresis, and in which enzyme was omitted. Significant quantities of radioactivity were found in the succinyl phosphate area only when succinate, ATP, and enzyme were present in the reaction mixture. When the succinyl phosphate area was eluted with water and the eluate was heated at 100° for 3 minutes, electrophoretic study showed that virtually all of the ^{32}P moved in the inorganic phosphate area. No succinyl phosphate was formed when enzyme was omitted, and much smaller amounts of radioactivity (or no radioactivity) were found in this area when succinate was omitted. When deproteinized samples of reaction mixtures containing ATP, succinate, and enzyme were treated with hydroxylamine, prior to electrophoresis, little or no radioactivity appeared in the succinyl phosphate area.

TABLE III: Evidence for Enzymatic Synthesis of Succinyl Phosphate.

Expt	Reaction Mixtures[a]	Succinyl Phosphate Formed (mμmoles)
1	Enzyme (780 units) + succinate + ATP	0.78
	Enzyme (780 units) + ATP	0
2	Enzyme (685 units) + succinate + ATP	1.02
	Enzyme (685 units) + succinate + ATP + NH_2OH[b]	0.02
	Enzyme (685 units) + ATP	0.05
3	Enzyme (274 units) + succinate + ATP	0.32
	Enzyme (274 units) + ATP	0.08
4	Enzyme (685 units) + succinate + ATP	0.78
	Enzyme (685 units) + ATP	0.06
5	Enzyme (685 units) + succinate + ATP	0.76
	Enzyme (685 units) + succinate[c] + ATP	0.17
	Enzyme (685 units) + ATP	0.07

[a] The reaction mixtures contained enzyme as indicated, Tris-HCl (*p*H 7.4, 20 μmoles), $MgCl_2$ (1 μmole), 2-mercaptoethanol (2 μmoles), sodium succinate (20 μmoles), and [γ-^{32}P]ATP (58 mμmoles; 1.3×10^6 cpm) in a final volume of 0.2 ml. The components were mixed at 0° (enzyme was added last) and the mixtures were placed at 20° for 45 seconds. Absolute ethanol (0.21 ml) was then added followed by 0.01 ml of carrier 0.17 M succinyl phosphate. After centrifugation, 0.04-ml aliquots of the supernatant solutions were subjected to paper electrophoresis as described under Experimental. [b] A sample (0.15 ml) of the ethanolic supernatant solution was mixed with 0.015 ml of 8 M NH_2OH and allowed to stand at 0° for 10 minutes; 0.04 ml was used for electrophoresis. [c] Succinate, 2 μmoles.

Incorporation of ^{32}P from [^{32}P]Succinyl Phosphate into a Phenol-soluble Form. When the enzyme was incubated for 1 or 2 minutes with [^{32}P]succinyl phosphate significant amounts of radioactivity were converted into a phenol-soluble form (Table IV). The procedure used for phenol extraction is that employed by Bieber *et al.* (1964) in studies in which it was shown tht phosphate was incorporated into the enzyme as an *N*-phosphorylimidazole derivative. In the present studies, it was found that incorporation of radioactivity was substantially greater with succinyl phosphate than with preparations of succinyl phosphate that were heated at 100° for 2 minutes prior to the experiment. Phosphorylation of the enzyme (as determined in this manner) was considerably reduced when coenzyme A, succinyl coenzyme A, or ADP was added.

Discussion

The data indicate that incubation of the enzyme with succinyl phosphate and coenzyme A leads to succinyl coenzyme A formation, and that ATP is formed when succinyl phosphate, ADP, and enzyme are incubated. The additional finding that the enzyme can synthesize succinyl phosphate from ATP and succinate (Table III) provides substantial reason to consider enzyme-bound succinyl phosphate as an intermediate in the reaction. Earlier attempts to demonstrate the utilization or formation of succinyl phosphate were apparently unsuccessful (Hager, 1962; Kaufman, 1955); however, it is not clear whether conditions similar to those employed here were used. The present findings are consistent with the following reactions:

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

$$\text{enzyme–succinyl phosphate} + \text{coenzyme A} \rightleftharpoons \text{enzyme} + \text{succinyl coenzyme A} + P_i \quad (2)$$

Such an interpretation is consistent with the observation that phosphate oxygen is incorporated into succinate oxygen in the course of the reaction. However, the recent observation that succinyl coenzyme A synthetase

TABLE IV: Incorporation of ^{32}P from [^{32}P]Succinyl Phosphate into a Phenol-soluble Form.

Reaction Mixtures[a]	Incubation Time (min)	Phosphorus in Phenol (mμmoles) With Succinyl Phosphate	Phosphorus in Phenol (mμmoles) With Heated Succinyl Phosphate[b]
No enzyme	1 or 2	0.01	0.01
Enzyme (950 units)	1	1.66	0.13
	2	2.05	0.22
Enzyme (950 units), CoA (0.013 μmole)	1	0.26	0.06
	2	0.44	0.07
Enzyme (1900 units)	1	1.91	0.16
	2	3.03	0.25
Enzyme (1900 units), CoA (0.013 μmole)	1	0.41	0.06
	2	0.93	0.07
Enzyme (1900 units), CoA (0.13 μmole)	1	0.08	0.03
Enzyme (1900 units), succinyl CoA (0.1 μmole)	1	0.05	0.10
Enzyme (1900 units), ADP (1.0 μmole)	1	0.08	0.05
Enzyme (1900 units), CoA (0.013 μmole), ADP (1.0 μmole)	1	0.08	0.12

[a] The reaction mixtures contained enzyme (in the amounts indicated), Tris-HCl buffer (*p*H 7.4, 30 μmoles), $MgCl_2$ (1.5 μmole), 2-mercaptoethanol (3 μmoles), [^{32}P]succinyl phosphate (32 mμmoles, 2.4×10^6 cpm), CoA, succinyl CoA, and ADP as indicated, in a final volume of 0.3 ml. The components were mixed at 0° (enzyme was added last) and incubated at 37° for 1 or 2 minutes as indicated. The reaction was stopped by addition of 1.0 ml of liquid phenol. The phenol layer was extracted with 2.5- to 3.0-ml portions of potassium phosphate (0.01 M, *p*H 7.8, saturated with phenol) until the radioactivity in the aqueous layer was constant. The radioactivity of the phenol layer was then determined. [b] Heated at 100° for 2 minutes.

can be phosphorylated by incubation with succinyl coenzyme A and inorganic phosphate, and also by incubation with ATP, suggests that the mechanism of the reaction is more complicated than indicated by equations (1) and (2). Phosphorylation of the enzyme might occur by reaction of ATP with the enzyme as follows:

$$\text{enzyme} + \text{ATP} \rightleftharpoons \text{enzyme-phosphate} + \text{ADP} \quad (3)$$

Phosphorylated enzyme might react with succinate as follows:

$$\text{enzyme-phosphate} + \text{succinate} \rightleftharpoons \text{enzyme–succinyl phosphate} \quad (4)$$

It is of considerable interest that the enzyme is readily phosphorylated by succinyl phosphate, and that such phosphorylation is reduced by addition of either coenzyme A or ADP (Table IV). This observation gives further support to a mechanism involving succinyl phosphate as an enzyme-bound intermediate, and hence to a series of reactions indicated by equations (3), (4), and (2).

The present findings do not exclude the occurrence of additional reactions, e.g., binding of coenzyme A to the enzyme, nor do they unequivocally rule out mechanisms not involving succinyl phosphate. It is conceivable that the enzyme can catalyze reactions which do not lie on the major catalytic pathway. Boyer and colleagues[3] have obtained preliminary evidence for an "activated" form of the enzyme, which may contain coenzyme A. Mechanisms involving anhydride formation between an enzyme carboxyl group and phosphate (as well as succinate) would be consistent with the ^{18}O data (see Falcone and Boyer, 1959), but there are apparently no published data indicating the existence of such forms of the enzyme. In the light of the positive results obtained with chemically synthesized succinyl phosphate and the demonstration that the enzyme can synthesize succinyl phosphate, we are inclined to favor the succinyl phosphate pathway.

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CORRECTION

In the paper by D. C. Sharma and R. I. Dorfman in Volume 3, No. 8, August 1964, on p. 1097, column 1, the last line of the next-to-last paragraph should read, "from progesterone."